**Universidade Nova de Lisboa**

**Instituto de Higiene e Medicina Tropical**

# Evaluation of humoral and cellular immune responses to *P. berghei*-based whole-sporozoite malaria vaccination in rhesus macaques

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# Resumo

A malária é uma doença infecciosa transmitida por mosquitos que continua a ser uma das doenças infecciosas mais impactantes do mundo, matando milhares de pessoas todos os anos. Apesar de todos os esforços para controlar esta doença, como quimioprofilaxia e medidas de controlo de vetores, a falta de conhecimento sobre as respostas imunes desencadeadas pelo parasita *Plasmodium* dificulta o desenvolvimento de uma vacina eficaz contra a malária, que é necessária com urgência.

A natureza assintomática e altamente imunogénica do estadio hepático da infecção por *Plasmodium* torna-o num alvo ideal para o desenvolvimento da vacina contra a malária. Prudêncio lab do Instituto de Medicina Molecular (IMM) desenvolveu um novo candidato a vacina contra a malária de esporozoito inteiro que tem como alvo o estágio hepático, a *Pb*VAC. Na qual o parasita roedor *P. berghei* expressa a proteína circunsporozoítica de superfície do *P. falciparum* (*Pf*CSP), altamente imunogénica, de modo a promover respostas imunitárias específicas para *Pf*CSP, assim como respostas cruzadas entre espécies, que podem proteger contra uma infecção subsequente por *P. falciparum*.

Estudos pré-clínicos em modelos de infecção em murganhos e coelhos mostraram que *Pb*Vac é capaz de infectar e desenvolver-se em hepatócitos sem estabelecer uma infecção no estadio sanguíneo. Para imitar o que acontece no fígado humano, os macacos rhesus (*Macaca mulatta*) foram usados para um teste pré-clínico de *P. berghei wild-type* (WT) e esporozoítos *Pb*Vac geneticamente modificados, como estratégia para induzir imunidade contra *P. falciparum*. Este estudo tem como objetivo investigar e comparar as respostas imunes humorais e celulares entre animais imunizados e não imunizados.

Primeiro, confirmamos que os esporozoítos do *Pb*WT são capazes de infectar hepatócitos do macaco rhesus in vivo. Posteriormente, os macacos rhesus foram imunizados por picada de mosquito com *Pb*Vac ou *Pb*WT e seguidos por 21 semanas, em paralelo com animais não imunizados. Células mononucleares do sangue periférico (PBMCs) e plama foram coletadas periodicamente e células do fígado e esplenócitos foram coletados na eutanásia. Foi realizada uma comparação do plasma pré e pós 3 imunizações para analisar as respostas humorais quantificando IgGs específicas para esporozoítos. As composições dos compartimentos imunes do sangue periférico, fígado e baço foram analisadas por imunofenotipagem e respostas imunes celulares específicas contra esporozoítos *Pb*VAC, *Pb*WT e *Pf* foram avaliadas em PBMCs e células hepáticas utilizando um ensaio intracelular de citocinas.

As imunizações foram seguras, sem alterações relevantes nos parâmetros de segurança avaliados, e nenhuma infecção relevante foi encontrada. Mostramos que as imunizações contra *Pb*WT e *Pb*Vac são capazes de provocar uma resposta humoral contra o antigénio em macacos. É importante ressaltar que a geração de anticorpos anti-esporozoítos *Pf* observados em animais imunizados com *Pb*Vac- mas não com *Pb*WT indica que a *Pf*CS pode desempenhar um papel significativo nas respostas humorais à vacina.

Quanto ao papel da imunidade celular desencadeada por essas imunizações, nem o perfil fenotípico geral, nem a magnitude e especificidade das respostas celulares aos agentes de imunização ou a *Pf* foram significativamente alteradas após a imunização. Em

contraste com o que alguns ensaios em humanos relataram, não encontramos expansão da população de células T γδ após a imunização. No entanto, encontramos alterações fenotípicas nas células linfóides inatas (ILCs), que diminuíram significativamente, e um aumento nas células T CD4+ nos PBMCs.

No geral, os nossos resultados indicam que a imunização com *Pb*Vac representa uma plataforma de vacinação segura que gera respostas imunes humorais a *Pf*. Manipulação adicional da estratégia de imunização com PbVac, como dose ou modo de administração, pode melhorar significativamente as suas respostas imunológicas humorais e celulares, contribuindo assim para o desenvolvimento de uma vacina eficiente contra a malária.

Palavras-chave: *Pb*Vac; vacina; reação de espécies cruzada; immunização; imunidade humoral; imunidade celular.

# Abstract

Malaria is a mosquito-borne infectious disease that remains one of the most impactful infectious diseases globally, killing thousands of people every year. Despite all efforts to control this disease, such as chemoprophylaxis and vector control measures, the lack of knowledge about the immune responses triggered by the *Plasmodium* parasite hinders the development of an urgently needed efective malaria vaccine.

The asymptomatic and highly immunogenic nature of the liver stage of *Plasmodium* infection makes it an ideal target for malaria vaccine development. The Instituto de Medicina Molecular (IMM)'s Prudêncio lab has developed a new pre-erythrocytic whole-sporozoite malaria vaccine candidate, *Pb*Vac, in which the rodent *P. berghei* parasite expresses the highly immunogenic *P. falciparum* surface circumsporozoite protein *(Pf*CS in order to promote *Pf*CS-specific and cross-species immune responses that may protect against a subsequent *P. falciparum* infection.

Pre-clinical studies in mouse and rabbit models of infection have shown that *Pb*Vac is able to infect and develop in hepatocytes without establishing a blood stage infection. In order to mimic what happens in the human liver, rhesus macaques (*Macaca mulatta*) were used for a pre-clinical analysis of *P. berghei* wild-type (WT) and genetically modified *Pb*Vac sporozoites, as a strategy to induce immunity *to P. falciparum*. This study aims to investigate and compare the humoral and cellular-associated immune responses between immunized and non-immunized animals.

First, the ability of *Pb*WT sporozoites to infect rhesus macaque hepatocytes *in vivo* was confirmed. Subsequently, rhesus macaques were immunized by mosquito bite with *Pb*VAC or *Pb*WT and followed for 21 weeks, in parallel with non-immunized animals. Peripheral blood mononuclear cells (PBMCs) and plasma were collected periodically, and liver cells and splenocytes were collected at euthanasia. A comparison between plasma pre- and post- 3 immunizations was performed to analyze humoral responses through quantification of specific IgGs for sporozoites. The compositions of the peripheral blood, liver and spleen immune compartments were analyzed by immunophenotyping, and specific cellular immune responses against *Pb*VAC, *Pb*WT and *Pf* sporozoites were assessed in PBMCs and liver cells by an intracellular cytokine assay.Immunizations were safe, with no relevant changes in the safety parameters evaluated, and no breakthrough infections were found. We show that both *Pb*WT and *Pb*Vac immunizations are capable of eliciting a humoral response against the immunogen in monkeys. Importantly, the generation of anti-*Pf* sporozoites antibodies observed in *PbVac*- but not *PbWT*-immunized animals indicates that *Pf*CS may play a significant role in humoral responses to the vaccine.

As for the role of cellular immunity elicited by these immunizations, neither the overall phenotypic profile nor the magnitude and specificity of cellular responses to the immunization agents or to *Pf* were significanty altered upon immunization. In contrast to what some human trials have reported, we found no expansion of the γδ T cell population upon immunization. However we found phenotypic changes in innate lymphoid cells ILCs, which decreased significantly, and an increase in CD4+ T cells in PBMCs.

Overall, our results indicate that *Pb*Vac immunization represents a safe vaccination platform that generates humoral immune responses to *Pf*. Additional manipulation of the

*Pb*Vac immunization strategy, such as dose or mode of administration, may significantly enhance its humoral as well as cellular immune responses, thus contributing to the development of an efficient malaria vaccine.

Keywords: *Pb*Vac; vaccine; cross-species immunization; humoral immunity; cellular immunity.

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# Abbreviation

Acid-citrate-dextrose (ACD)

Acute respiratory distress syndrome (ARDS)

Alanine transaminase (ALAT)

Allophycocyanin ef780 (apcef780)

Antibody-dependent cellular cytotoxicity (ADCC)

Antigen-presenting cells (APC)

Apical membrane antigen 1 (AMA-1)

Artemisinin-based combination therapy (ACT)

Aspartate transaminase (ASAT)

Biomedical Primate Research Centre (BPRC)

Blood-stage vaccines (BSV)

Central memory T cells ($T_{CM}$)

Cerebral malaria (CM)

Chemokine receptor 7 (CCR7)

Chemokine receptor type 5 (CXCR5)

Chloroquine chemoprophylaxis sporozoites (CPS)

Circumsporozoite protein (CSP)

Controlled human malaria infection (CHMI)

Dendritic cells (DCs)

Dimethyl sulfoxide (DMSO)

Double negative (DN)

Effector memory cells ($T_{EM}$)

Enzyme-linked immunosorbent assay (ELISA)

Ethylenediaminete traacetic acid (EDTA)

Exo-erythrocytic forms (EEFeefs)

Fix/Perm (fixation/permeabilization

Fixable viability dye (FVD)

Fluorescein isothiocyanate (FITC)

Follicular cytotoxic T cells (TFC)

Follicular helper T cells (Tfh)

Forward scatter (FSC)

Gamma glutamyl transpeptidase (γ-GT)

Gene insertion/marker out (GIMO)

Genetically attenuated sporozoites (GAS)

Heat shock protein 70 (HSP70)

Helper T cells (Th)

Heparan sulphate proteoglycans (HSPGs)

Hepatitis B surface antigen (hbsag)

Horseradish peroxidase (HRP)

Human leukocyte antigen DR (HLA-DR)

Immunoglobulins (Ig)

Indoor residual spraying (IRS)

Innate lymphoid cells (ilcs)

Insecticide treated bed-nets (ITN)

Interferon- γ (IFN-γ),

Interleukin- 2 (IL-2)

Intracellular cytokine assay (ICS)

Intradermally (id)

Intravenous (iv)

Knock out (KO)

Lactate dehydrogenase (LDH)

Lipopolysaccharide (LPS)

Major histocompatible complex (MHC)

Malaria Vaccine Implementation Programme (MVIP)

Mean corpuscular volume (MVC)

Median fluorescence intensity (MFI)

Merozoite surface protein (MSP)

Myeloid DCs (mDCs)

Natural killer (NK)

Natural killer T (NKT)

New Zealand White (NZW)

Nitric oxide (NO)

Nonhuman primates (NHP)

*P. Berghei* (*Pb*)

*P. Falciparum* (*Pf*)

Parasitophorous vacuole (PV)

Parasitophorous vacuole membrane (PVM)

Pathogen-associated molecular patterns (PAMPs)

Pattern recognition receptors (PRRs)

*Pf*spz (*P. Falciparum* sporozoite),

Phorbol myristate acetate (PMA)

Phosphate-buffered saline (PBS)

Phycoerythrin (PE)

Plasmacytoid dcs (pdcs)

*Plasmodium* (P.)

Pre-erythrocytic vaccines (PEV)

Radiation-attenuated sporozoites (RAS)

Rapid diagnostic tests (RDT)

Reactive oxygen species (ROS)

Red blood cells (RBC)

Regulatory T cells (Treg)

Room temperature (RT)

Roswell Park Memorial Institute (RPMI)

Side scatter (SSC)

Sporozoite protein essential for cell traversal (SPECT)

Subcutaneously (sc)

T cell receptor (TCR)

Tissue-resident memory T cells ($T_{RM}$)

TMB (Tetramethylbenzidine)

Transmission-blocking vaccines (MSTBV)

Tumor necrosis factor (TNF)

Upregulated in infective sporozoites (UIS)

Whole sporozoite (Wsp)

World Health Organization (WHO)

A-galactosylceramide (α-galcer)

# Introduction

## 1.1. Malaria

Malaria is one of the most impacting infectious diseases worldwide, especially in tropical and subtropical regions of the world. According to estimates from the World Health Organization (WHO), 219 million cases of malaria occurred worldwide in 2017, compared with 216 million in 2016. Of these cases, 92% were registered in the WHO African region. In that same year, 435 000 deaths were estimated from malaria globally, and 61% of them occurred in children under 5 years of age[1].

Malaria is a mosquito-borne parasitic disease, caused by a protozoan parasite, phylum *Apicomplexa* and gender *Plasmodium* (P.) and transmitted through the bite of a female A*nopheles* mosquito[2]. The species capable of causing infection in humans are: *P. falciparum*, the major responsible for sub-Saharan Africa cases, complicated malaria and mortality; *P. vivax*, the most prevalent in America WHO regions; *P. ovale*; *P. malariae*; and *P. knowlesi*, primarily known as a primate parasite, but recently identified in humans in Southeast Asia[3,4]. Other *Plasmodium* species are able to infect different hosts, such as *P. berghei* and *P.yoelii* in rodents and *P. knowlesi*[3] and *P. simium* in non-human primates[5]. Their ability to mimic the human infection renders them important for malaria research.

### 1.1.1 **Plasmodium life cycle**.

The malaria parasite presents a very complex, multistage life cycle involving a mosquito and a mammalian host (Fig. 1). After their entry into the host via mosquito bite, Plasmodium parasites enter the circulation and reach the liver. The liver stage is obligatory, asymptomatic, and induces an immune response, thus constituting an ideal target for anti-malarial vaccines and prophylactic drug development6. Parasites released into the bloodstream subsequently infect red blood cells (RBC) and massive replication occurs, initiating the stage responsible for malaria symptoms and transmission.

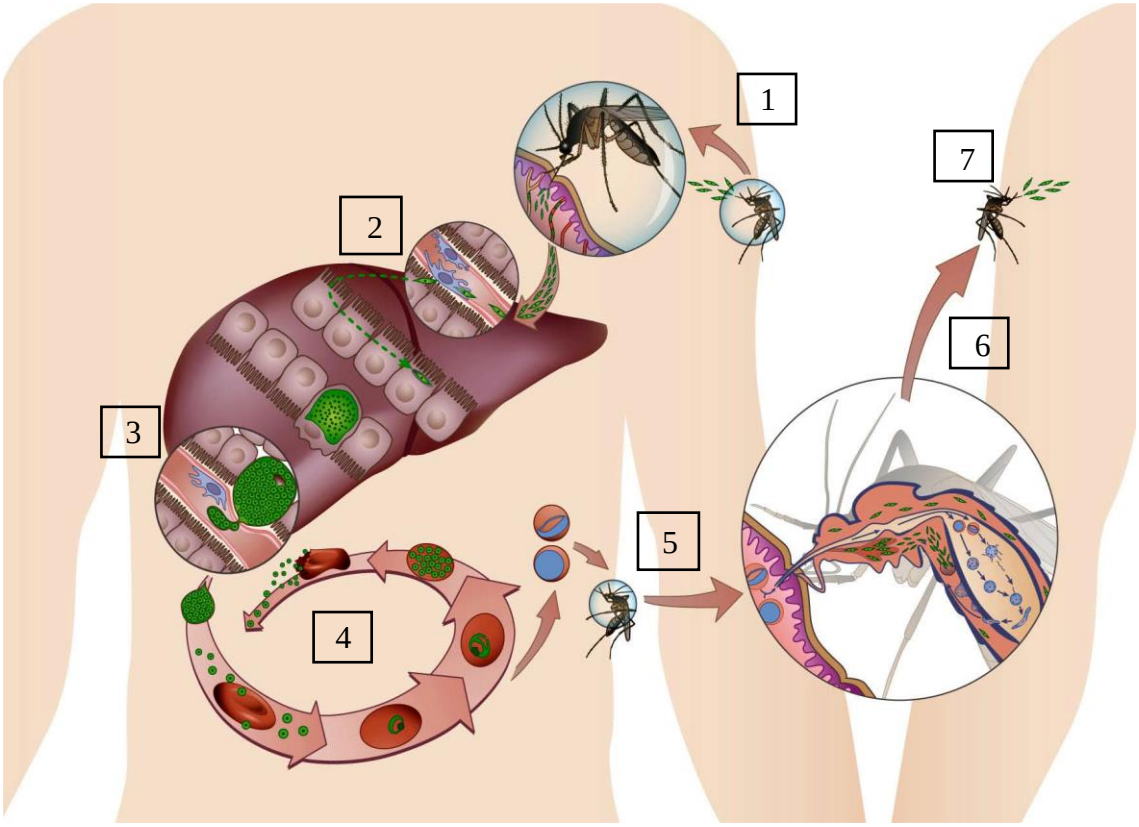


*Figure 1- Plasmodium spp.* Life cycle. (1) An infected female Anopheles mosquito injects sporozoites into a mammalian host. This liver-infective parasite forms will travel until reaching the liver. (2) Once in the liver, the parasite transverses several hepatocytes until establishing a productive infective in one, where a PV is formed. An intense asexual reproduction by schizogony occurs to form thousands of merozoites inside vesicles. (3) These merosomes bud off from the hepatocyte and are released into the bloodstream. (4) In the RBC the parasite performs successive cycles of invasion, intracellular growth, proliferation and re-invasion. (5) Some parasites are sexually committed merozoites, that develop into gametocytes that are able to develop within the mosquito when a new blood meal takes place. (6) Inside the vector, the parasite undergoes several transformations, formation of diploid zygotes, division and maturation into motile ookinetes, which are able to penetrate the midgut wall and transform into oocysts. The sporozoite formation initiates, and the accumulation of thousands of mature sporozoites causes the disruption of the oocysts and the release of sporozoites that invade salivary glands. (7) The sporozoites attached to the salivary glands are ready to be injected in the dermis of a new mammalian host.

The *Plasmodium* life cycle is initiated when an infected mosquito bites the mammalian host to take a blood meal and injects hundreds of sporozoites, the liver-infective forms, into the host's skin[7]. Once in the skin, the parasite starts moving

randomly, in a gliding motility through the cells and tissues of the host[8], to reach the blood vessels[9,10]. Most of these *Plasmodium* parasites actively target the liver (Fig. 1.1), possible due to the recognition and binding of the circumsporozoite protein (CSP), which covers the surface of the parasite, to the negatively charged heparan sulphate proteoglycans (HSPGs) expressed on the surface of the hepatocytes[6]. Due to its involvement in sporozoite motility as well as hepatocyte invasion and infection, CSP is an important target for drugs and prophylaxis in a pre-erythrocytic phase[11,12]. Once sporozoites reach the liver, they circulate freely along the sinusoidal epithelium, and transverse endothelial and Kupffer cells and several hepatocytes. Sporozoite protein essential for cell traversal (SPECT) and SPECT2 are two sporozoite proteins of micronemes identified in rodent parasite models that play a crucial role in cell transversal activity and liver infection[13].

After migrating through several hepatocytes, the sporozoite establishes a productive infection in one[14], where a parasitophorous vacuole (PV) is formed and asexual reproduction (schizogony) and maturation takes place (Fig. 1.2). Three rodent PV proteins, Upregulated in infective sporozoites (UIS)3, UIS4 and *Pb*36p, have been identified as being essential for parasite liver stage development within the PV[6,12]. The end of the exoerythrocytic phase is marked by the formation of thousands of merozoites that are released into the bloodstream inside vesicles, termed merosomes, derived from the host cell to deceive the immune system (Fig. 1.3)[15]. This exoerythrocytic stage is characteristic of each species and takes a minimum of 6 days in case of infection by *P. falciparum* and until a maximum of 16 days for P. *malariae* [2], and only 2 days for the rodent-infective parasites (*P. berghei and P.yoelii*)[16]

Merozoites have the capacity to infect RBCs and perform successive cycles of invasion, intracellular growth, proliferation and re-invasion. The invasion step occurs quickly and involves multiple recognitions and interactions[17], from connection of the merozoite to the RBC to its reorientation, which are mediated by the large merozoite surface protein (MSP) and the apical membrane antigen 1 (AMA-1), respectively. Then, merozoite penetration occurs by different parasite transmembrane protein families and at the same time a parasitophorous vacuole membrane (PVM) is formed with its own plasma membrane[17,18].

The initial form of the erythrocytic cycle is the ring stage, when the parasite begins to feed on the surrounding RBC[19], and develops to trophozoite stage, when the synthesis of molecules needed for cell division occurs[20,21]. During this stage, *P. falciparum*-infected RBC have the capacity to adhere to the endothelium of capillaries and venules and to non-infected RBC[22]. This sequestration is suggested to be a parasite defence mechanism to avoid splenic depuration and can lead to serious problems, such as cerebral malaria[23]. Lastly, a replicative intra-erythrocytic plasmodium stage, termed schizont, leads to the formation of around 16 nuclei that constitute a merozoite. The rupture of the PV and RBC membranes allows the merozoites to be released into the bloodstream and enables them to invade a new RBC and restart the intra-erythrocytic cycle (Fig. 1.4)[20]. The erythrocyte burst and parasites release is the cause of fever spikes that differ in the number of days according the infective species. For example, *P. falciparum* peaks occur every third day[24].

Parasites can also develop into sexual stages, named gametocytes. These are a sexually committed merozoites that invade the RBC following the ring stage and subsequently grow and develop over five stages until becoming sexually differentiated gametocytes[25]. Only mature sexual stages are able to survive and continue the Plasmodium life cycle in the mosquito host, known as the sporogonic cycle, upon the blood meal of a female anopheles (Fig. 1.5)[26]. Ingestion of gametocytes stimulates gametogenesis that starts with gametes formation and transformation into fertile female macrogametes and male microgametes. Microgamete exflagellation initiates the fertilization process, which includes fusion of the two gametes[27]. Fertilization gives rise to a zygote, and meiosis and genetic recombination transform the zygote into a motile ookinete able to penetrate and attach in the midgut wall and develop into oocysts, where occur the CSP formation. Once the oocyst is full of mature sporozoites, it ruptures and release sporozoites into the haemolymph circulation to reach and attach in the salivary glands (Fig. 1.6). At this point, the parasites are infectious and able to be inoculated in the skin of the next mammalian host and reinitiate the cycle (Fig. 1.7)[12].

### 1.1.2. Clinical presentation and treatment

Malaria may have different clinical presentations, since absence of symptoms or very mild symptoms, to severe disease or even death. Therefore, malaria disease is

classified as uncomplicated or severe, and its severity may be dependent of the infecting species and on the host's immune status[28].

The gold-standard diagnosis of malaria are rapid diagnostic tests (RDT), which detect parasite antigens in the blood, or microscopy, that allows the quantification and identification of *Plasmodium* Spp. [29].

The initial symptom of the disease is usually fever due the merozoite bursting during RBC reinfection cycle and can appear 7 days after the infection[30]. Other symptoms include chills, sweats, headaches, nausea and vomiting, mild jaundice, body aches and general malaise, which can be confused with many other diseases, principally in non-endemic countries[30,31]. The progress of the symptoms is normally quick and, according to WHO, patients with one or more established clinical or laboratory features are considered to have severe malaria, and therefore treated as such[30]. Severe malaria has quite an extensive list of manifestations, the most common being cerebral malaria (CM), caused by sequestration of trophozoite-infected RBC into the blood vessels that can lead to coma and neurologic abnormalities, and severe anaemia, due to the destruction of RBCs. Other clinical manifestations include haemoglobinuria, acute respiratory distress syndrome (ARDS), blood coagulation abnormalities, low blood pressure, acute kidney injury and hyperparasitemia (more than 5% of RBCs infected by malaria parasites), metabolic acidosis, hypoglycaemia and death[31].

The WHO provides some guidelines for malaria control relative to vector control trough the indoor residual spraying (IRS) and sleeping under insecticide treated bed-nets (ITN), and chemoprevention in pregnant women, children and travellers from non-endemic countries. However, an effective malaria vaccine would be most efficient way to control this infectious disease.[1]

Despite the significant work done towards reducing the burden of malaria, with much progress made, millions of malaria cases occur every year worldwide and need to be treated to prevent the progression of the disease, transmission and the resistance of antimalarial drugs[1]. According to WHO guidelines, artemisinin-based combination therapy (ACT) is the best treatment for *P. falciparum* infection in adults and children. ACT combines a rapidly active artemisinin derivative, that rapidly clears the major parasites of the blood, with a longer-acting compound, that clears the remaining parasite

and protects against development of artemisinin resistance forms. For the treatment of uncomplicated malaria caused by the remaining species, the recommended treatment is ACT or chloroquine, depending on the resistance profile of the area. *P. ovale* and *P. vivax* infection have the particularity to form dormant liver stage forms and relapse following the cure of the primary infection. Some sporozoites do not initiate their development in hepatocytes at the same time and remain quiescent in the liver for long periods (a form known as hypnozoites), eventually developing and causing recurrent infection[32].To prevent *P. vivax* and *P. ovale* relapses, the treatment is accompanied with primaquine. For severe malaria, the treatment is intravenous or intramuscular artesunate for at least 24h, and when the patient can tolerate oral therapy, switches to ACT treatment[29].

## 1.2. Malaria Immunology

### 1.2.1. Basic concepts in immunology

Immunology is the branch of biology that studies the immune system, including host defence against external and internal aggressions, elimination of pathological microbes and toxic or allergenic proteins and discrimination between self and nonself[33]. The immune system is composed of molecules and cells and essentially involves two components, the innate and the adaptative immune system. The innate immune system, although not pathogen-specific, constitutes the first line of defence against external insults and is characterized by a very quick response (minutes to hours). The adaptative immune system comprises a slow response, that can take days or longer, and forms immunological memory, in order to develop a specific and faster response against reinfection by the same pathogen[34].

The innate immune system includes physical barriers, defence mechanisms and general immune responses that aim to keep pathogens and foreign particles out of the body and are activated by the presence of antigens. Among the main functions of the innate system are the discrimination between self and nonself, recruitment of immune cells to the site of infection by producing chemical mediators, promoting the elimination of dead cells or antibody complexes, identification and removal of foreign elements by specialized white blood cells and activation of the adaptive immune system through

antigen presentation[35]. Some cells that are part of the innate immune system are phagocytic cells, including dendritic cells, as well as natural killer (NK) cells and natural killer T (NKT) cells. Pathogens are recognized by conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs), which are expressed on innate immune cells[35].

Monocytes, macrophages and dendritic cells (DCs) are phagocytic cells, which can function to ingest and eliminate pathogens and apoptotic cells, as well as to present antigen (antigen-presenting cells, APC), leading to T cell activation and cytokine production[36]. They express human leukocyte antigen DR (HLA-DR), a major histocompatible complex (MHC) class II cell surface receptor, to present peptide antigens from extracellular proteins. In humans, monocytes express CD14 ("classical" monocytes), a lipopolysaccharide (LPS)-binding protein, which functions as an endotoxin receptor anchored to the cell surface, and can express the Fcγ receptor III (FcγRIII or CD16; "non-classical" monocytes)[37,38]. Immature dendritic cells reside in the peripheral tissues and, upon antigen encounter, migrate to primary and secondary lymphoid organs to present antigens to naïve T cells. This is mainly performed by myeloid DCs (mDCs), which express CD11c (integrin alpha X), a transmembrane protein that induces cellular activation. Another type of DCs, the plasmacytoid DCs (pDCs), are also known as natural type-I-interferon-producing cells and express interleukin-3 receptor (CD123)[39].

Innate lymphoid cells (ILCs) are a recently discovered immune population that presents a quick response to infection and secrete inflammation mediators similarly to T lymphocytes. This type of cells don't express antigen receptors and can be divided based on the cytokines that they can produce, and the transcription factors that regulate their development and function[40]. ILCs are usually defined by the absence of lineage markers, as well as the expression of the leucocyte common antigen CD45 and of the interleukin-7 receptor subunit alpha (CD127)[41].

NK cells, which are classified as group 1 ILCs, mature in the bone marrow and in secondary lymphoid organs and are essential for the innate immune response, since they recognize infected cells bearing low levels of of MHC. NK cells can directly lyse infected cells or can produce inflammatory cytokines and mediate regulatory functions of other

cell types[42]. In rhesus monkey NK cells present CD16 on their surface, which mediates antibody-dependent cellular cytotoxicity (ADCC) [37].

NKT cells are an immune cell population that compise features of both NK and T cells, which express an invariant αβ T cell receptor (TCRαβ) and recognize lipid antigens. This type of cells bridges the innate and the adaptive immune responses through secretion of large amounts of pre-formed cytokines upon activation, leading to downstream recruitment and activation of DCs, NK, CD4+ and CD8+ T cells[43],[37].

Professional APCs presenting peptides through their MHC class I or II promote antigen recognition by the TCRαβ and lead to proliferation, maturation and differentiation of naïve CD8+ or CD4+ T ($T_N$) cells, respectively, into memory cells[44]. Memory cells can recirculate through lymphoid (central memory cells, $T_{CM}$) or non-lympoid (effector memory cells, $T_{EM}$) tissues, or they may reside in tissues and monitor perturbations in homeostasis, such as infection (tissue-resident memory T cells, $T_{RM}$)[45]. Naïve and memory cells can be broadly identified by the expression of chemokine receptor 7 (CCR7) and CD95 the apoptosis antigen 1 or Fas, respectively[46]. $T_{RM}$ express the early activation marker CD69 and produce pro-inflammatory cytokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin 2 (IL-2) upon reactivation[45].

Upon activation, CD8 T cells differentiate into cytotoxic T cells, which kill infected cells[47,48]. CD4 T cells may develop into distinct sub-populations that are key mediators of the immune response, such as: helper T (Th) cells, regulatory T cells (Treg), follicular helper T cells (Tfh) or follicular regulatory T cells (Tfr). Each CD4+ Th subset releases specific cytokines that can have either pro- or anti-inflammatory functions, survival or protective functions. For example, Th1 cells produce IFN-γ, TNF-α and IL-2, while $T_{H2}$ cells produce IL-4, IL-5 and IL-13[49]. Treg supress proliferation of other lymphocytes and thus modulate immune homeostasis, including autoimmune reactions and chronic infections. They are characterized by high IL-2 receptor α-chain (CD25) and CD127 expression, and rely on IL-2 generated by T cells during an immune response to perform their immune regulatory functions[50,51]. Tfh express the chemokine receptor type 5 (CXCR5), which enables them to migrate to lymphoid follicles, but not CD25[52], and provide help to B cells[53]. They are essential for germinal centre formation and for

antibody affinity maturation and isotype switching, and thus for the generation of high affinity antibodies involved in protective immunity against pathogens[53]. Tfr share characteristics with Tfh and Treg cells and participate in the regulation of GC reactions[54]. CD8+ follicular cytotoxic T cells (Tfc) constitute a CXCR5+ cytotoxic subpopulation located in, or proximal to B cell follicles in secondary lymphoid tissues that have been associated with elimination of virus-infected B cells and regulation of antibody responses[55].

A small but important subset of unconventional T cells expresses TCRγδ instead of TCRαβ. These so called γδ T cells respond to a variety of microbial pathogens and transformed/tumor cells in an MHC-independent manner, acting directly through their cytotoxic activity or indirectly through cytokine production[56].

Humoral immunity concerns the protection of extracellular spaces, which includes the antibody-mediated neutralization or opsonization of microbial molecules as well as the triggering of complement system activation[57]. Antibodies (or immunoglobulins, Ig) are produced by B cells, a type of lymphocyte that matures in the bone marrow and is activated in secondary lymphoid tissues. Once activated, naïve B cells form germinal centers where they proliferate, differentiate and undergo class-switch recombination, somatic hypermutation and affinity maturation in order to differentiate into antibody-producing plasma B cells and memory B cells[58,59]. Memory B cells recirculate through the body as resting lymphocytes until reactivation, in which case they develop a faster, stronger and more specific response to the antigen. B cells are generally identified by the B-lymphocyte antigen CD20, and naïve B cells feature membrane-bound IgD as well as CD21, the complement receptor type 2. Memory B cells can be identified by the expression of CD27, a member of the tumor necrosis factor (TNF) receptor that plays a key role in regulation and activation of B cells and contributes to immunoglobulin synthesis, and subdivided in terms of Ig expression into non-switched or switched (expressing IgG, IgA, or IgE) memory B cells[58].

### 1.2.2. Immunology in malaria infection

*Liver stage infection*

Innate immunity plays a critical role against the malaria pathogen, being the host's first line of defence. Studies in mice reveal that the innate immune response to *Plasmodium* developed at this stage is mediated by interferon type I (group of structurally similar cytokines including IFNα and IFNß) and type II (composed only by IFNγ, produced by activated lymphocytes)[60,61]. *Plasmodium* is known to induce a potent response mediated by IFN- γ whose main functions can influence destruction of *Plasmodium*-infected cells due to increasing cytotoxicity of CD8+ T cells, induction of B cells to produce cytophilic antibodies and enhancing phagocytic functions of immune cells such as macrophages[62]The IFN-mediated host resistance to reinfection explains the fact that in regions of malaria hyperendemicity, where people face regular and repeated reinfections, many of them do not translate into disease[63].Type-I interferon acts paracrinally to recruit lymphocytes to the liver, such as CD8+ cells, NKT and NK cells, and they are often found surrounding the infected hepatocytes[64]. NKT and NK are responsible for initiating cell-mediated immunity[64]. NKT cells contribute to avoid the parasite development in hepatocytes, NK control the parasite burden trough IFN-γ production, and CD8+ produce IFN-γ to limit parasite growth.[64] The role of NKT population in malaria infection was demonstrated by the inhibition of liver stage parasite development in mice that were administerated with α-galactosylceramide (α-GalCer), a glycolipid known to activate CD1d-restricted NKT cells, and infected with *Plasmodium* sporozoites. This inhibition was shown to be mediated through IFN-γ produced by the α-GalCer-activated NKT cells[65].

When the malarial antigens are presented to CD8+ or CD4+ T cells, CD8+ T cells induce the death of the infected hepatocyte via a perforin/granzyme B- or Fas-dependent mechanism[66]. In the mouse model, activated/memory T cells are important for protective immunity since in the short time the parasite is in the liver naïve CD8+ T cell proliferation and generation of effector functions is limited[67]. In humans, however, naïve parasite-specific T cells could contribute to the anti-parasite immune response, since they have sufficient time to be primed, expand and acquire effector functions[68]. Naïve CD8+ T cells will differentiate into short-lived effectors or memory precursor effector cells depending on several aspects, such as localization and transcription factors expressed by the cell.

The latter produce less effector proteins and can be observed throughout and after the infection and will develop into true memory T cells that provide long-term protection against their cognate antigen and rapidly gain effector functions upon reencounter[69].

$T_{RM}$ in malaria infection reside and patrol especially in the liver sinusoids and act as guards against pathogens and are poised to respond to an immediate threat[70]. After P. *falciparum* sporozoite immunization suppressing hepatic infection but also recruiting other effector cells to the liver[71] This type of cells express increased amounts of the effector molecules granzyme B, IFN-γ, and TNF-α, and despite their high importance in the liver, especially CD8+ $T_{RM}$ cells, they are also present in the spleen after sporozoite vaccination[70].

γδ T cells are one of the populations in the first line of defence against malaria infection capable of stimulating or suppressing immune responses to *Plasmodium* infection by inducing different immune cells. Their functions involve stimulation or repression of immune responses through distinct natural or induced cell subsets and help control primary *Plasmodium* infections in humans through the production of various immune mediators, such as IFN-γ, TNF-α or granzyme B. In humans this population appears to expand during acute, primary malaria infections and contract upon each reinfection, which may indeed help to control clinical malaria in endemic regions after several reinfections. It is hypothesized that this may be due to the decrease in the major γδ T cell subfamily, Vγ9Vδ2, with age and since in endemic regions most humans have been exposed to *Plasmodium* infection since childhood. Importantly, the severity of the primary disease increases with age, which may be due to the natural increase in γδ T cells[72]. It has also been suggested from vaccination experiments that γδ T cell depletion in mice prevents DC influx into the liver and prevents the existence of an effective CD8+ T cell response and consequent immunity to challenge however if this depletion occurs just prior to challenge there is no decrease in protection, suggesting the importance of γδ T cells in facilitating the immune response in the CD8+ T cell-mediated liver phase.[72] Experiments with γδ T cell-deficient C57BL/6 mice showed increased liver parasite (*P. yoelii*) burden, suggesting their contribution in the inhibition of the early intrahepatic parasite development[73]. On the other hand, γδ T cell-deficient C57BL/6 mice were reported not to develop experimental cerebral malaria upon infection with *Pb*ANKA, a

widely used model for cerebral malaria research, and this was likely due to the decrease in IFN-γ levels[74].

The role of antibodies produced by B cells is reflected from the moment that the sporozoites penetrate the skin. They are fit to inhibit sporozoite motility in both dermis and liver, improve phagocytosis in the spleen or liver by monocytes or macrophages, prevent connection between sporozoite ligand and hepatocyte receptor, and inhibit the development of the parasite inside the hepatocyte[75]. Antibodies can detect infected hepatocytes through specific proteins expressed on their surface and induce liver parasite killing [75]. Helper CD4 T cells help to stimulate the production of high levels of antibodies during natural infection[75].

### *Blood stage infection*

Parasitized erythrocytes do not present parasite antigens by MHC expression. Instead, parasites express antigens at the surface of iRBC erythrocytes and are indirectly target by CD4+ helper T cells and possibly γδ T cells that may orchestrate secreted antibody responses[72,76,75]. Parasite-specific antibodies potentially function to block merozoite invasion of RBCs, opsonize parasitized RBCs to enhance their phagocytosis and to target merozoites and infected RBCs for antibody-dependent cellular cytotoxicity[72,76,75].

CD8+ T cells play a less relevant role in blood-stage immunity, probably owing to the lack of MHC class I on erythrocytes, and there is no evidence that this population can inhibit blood-stage infection[75]. Despite its limited role at this phase, CD8+ T cells' response against *Plasmodium* erythrocytic stage antigens are primed primarily in the spleen, and according to mice studies, are involved in the pathogenesis of cerebral malaria[72]. This role is mediated directly by perforin and granzyme B and indirectly by the accumulation of parasitized RBC in the brain driven by IFN-γ[72]. Targeting of CD8+ T cells in an antigen-specific manner in cerebrovascular endothelial cells it is associated to blood–brain barrier dysfunction, subsequent vascular leakage and neuronal death[72].

## 1.3. Malaria vaccines

Vaccines are used to generate immunity against disease. They can contain the entire agent of the disease, attenuated or killed, or its toxins or surface proteins. The vaccine will promote the recognition of the antigen as foreign by the immune system and the generation of memory immune responses, for an easier and faster recognition and destruction of the pathogen upon a reencounter[77].

### 1.3.1. Overview of malaria vaccines

Vaccination is the most effective method to prevent infectious diseases, and the necessity of an efficient vaccine to control malaria is consensual. The complexity of the malaria parasite makes the creation of a vaccine a very complicated process. Despite all the efforts, there is no licenced antimalarial vaccine against the human-infective *Plasmodium* parasites[78].

*Plasmodium*'s complex life cycle allows the development of vaccines targeting different stages[79], such as: transmission-blocking vaccines (MSTBV) targeting the sexual stages, that impact on the parasite's life cycle in the mosquito vector aiming to prevent sporozoite development and onward transmission; pre-erythrocytic vaccines (PEV), inhibiting sporozoites infection, killing infected hepatocytes or inhibiting merozoites invasion; blood-stage vaccines (BSV), preventing RBC-mediated pathology and combined vaccines[80]. Most vaccines under pre-clinical or clinical development are for *P. falciparum* infection, but there are some liver stage vaccines in clinical trials for *P. vivax* infection[79]. Malaria vaccines can also be divided in whole sporozoite or subunit vaccines. Subunit vaccines consist in the delivery of a specific parasite antigen, normally with the use of an adjuvant to elicit immune protection against the parasite[81].

RTS,S/AS01 is the most advanced subunit malaria vaccine candidate to date. It uses *Pf*CSP fused with hepatitis B surface antigen (HBsAg). It is a PEV malaria vaccine that aims to prevent liver invasion or development of malaria parasites in the liver[82]. This vaccine shows 36% reduction in number of infections in children aged 5–17 months who receive three doses of the vaccine and then a booster at 20 months of age[83]. Therefore in 2016 WHO created a Malaria Vaccine Implementation Programme (MVIP) to introduce

this vaccine in three pilot countries (Ghana, Kenya and Malawi) and evaluate its impact and safety. After all this process, including evaluations and ethics approval, the vaccine, with the trade name Mosquirix, will be introduced in these three countries[84].

### 1.3.2. Whole organism pre-erythrocytic malaria vaccines

The liver stage is an ideal target for malaria vaccination and have shown the best results in antimalarial vaccines development. This stage is asymptomatic, present a low number of parasites, and it's metabolically and immunologically very active[15]. Whole organism vaccines consist in administering the entire organism, in this case the liver-infective form, sporozoites, to induce sterile immunity to the same species or a related specie[85]. The main whole sporozoite liver stage vaccines approaches are radiation-attenuated sporozoites (RAS), genetically attenuated sporozoites (GAS) and chloroquine sensitive sporozoites[86].

RAS causes a damage in the parasite DNA that blocks sporozoite replication[87]. The strategy of attenuating sporozoites by radiation as a malaria vaccine began when it was shown that it successfully elicited protection in mice against *P. berghei* through repeated intravenous injection or mosquito bite of X-irradiated sporozoites of the same species. Thereafter, in the 70's, the first clinical studies that showed that it is possible to achieve sterile immunity against *P. falciparum* and *P. vivax* with X-ray-attenuated sporozoites administrated by infected mosquito bites were performed. However, a very large number of mosquitoes was required to inoculate enough sporozoites to elicit protection, what was considered unsuitable for many years[88,89].

Hoffman et al. introduced a new concept of malaria vaccination consisting of immunization with gamma-attenuated sporozoites. The optimal radiation of sporozoite exposure was identified as 15.000 rad, with lower radiation being associated with breakthrough infections, and higher radiation with an over-attenuation of sporozoites. Protection was conferred with more than 1000 mosquito bites and lasted 9 weeks after last exposure against a primary controlled human malaria infection (CHMI) with *P. falciparum* and after rechallenge for at least 23–42 weeks[90]. To overcome the large number of mosquito bites involved, different administration routes were tested. The administration of aseptic, purified, cryopreserved whole-parasite malaria vaccine, termed

*PfSPZ* (P. falciparum sporozoite), was performed intradermally (id) in the forearm or subcutaneously (sc) in the upper arm to optimize the immunogenicity and protective efficacy of the method. In a dose-escalation study 7500, 30000 or 135000 *Pf*SPZ were administered in 4 or 6 doses, but only in 2 out of 80 volunteers revealed protection when challenged and all the immune response levels were low showing that the immunogenicity and protective efficacy of the vaccine were suboptimal, probably related to the administration route. Studies carried out in rhesus monkey led to a new clinical study with intravenous injection (iv) immunization to improve immunity and protection[91]. Another study in human volunteers showed the importance of the number of sporozoites, dose per volunteer and route of administration for an efficient malaria vaccine[92]. In this study lower immunization doses failed to establish a significant level of protection, while higher immunization doses showed efficient protection in of 6 out of 9 of the subjects of the 4-dose group and all of the volunteers of the 5-dose group[92]. In a recent clinical trial, three administrations of 900000 *Pf*SPZ conferred protection in 9 out of 14 volunteers against homologous CHMI[93]. Six of these 9 protected volunteers proceeded to a second CHMI with an heterologous parasite, and 5 of them remained without parasitaemia 33 weeks after the last immunization. In all the volunteers *Pf*SPZ-specific antibodies and T-cell responses were detected[93].

Recently, malaria vaccine candidates have been developed based on GAS, generated by removing some strategic genes upregulated in sporozoites and essential in the liver stage of the parasite[94,95]. However, some safety problems were reported for this approach. For example, the first human clinical trial with *P. falciparum* (genetically attenuated parasite) GAP *Pf*Δ*p52*Δ*p36,*generated by depletion of p52 and p36 (involved in the formation of the parasitophorous vacuole membrane) and administered by mosquito bite, had to be interrupted because of breakthrough infections in one volunteer during immunization[96]. This led to the creation of a triple gene deleted parasite by additionally removing the sap1 gene (*Pf* p52−/p36−/sap1−GAP), a cytoplasmic protein involved in regulating RNA stability and sporozoite gene expression. This triple knock out (KO) sporozoites are viable, infectious, don't develop completely in the liver stage and don't transit to blood stage infection[97].

Other GAS vaccine candidates, *Pb*Δ*b9*Δ*slarp* and the orthologue with equivalent *Pf* genes, have a depletion of two critical genes in the liver stage development identified

in *P. berghei*. B9 gene also belongs to 6-Cys family of *Plasmodium*, like p35 and p52, and SLARP/sap1 gene. The safety and efficacy evaluation of this GAS showed no breakthrough infections in *P. berghei*, and the human *Pb*Δb9Δslarp parasite was able to infect human hepatocytes *in vitro* as well humanized mice without developing, thus providing support for clinical development of a *Pf*Δb9Δslarp *Pf*SPZ vaccine[95].

Chloroquine sensitive sporozoites are another approach to achieve an efficient whole sporozoite pre-erythrocytic malaria vaccine. Chloroquine chemoprophylaxis with *Pf* sporozoites (CPS) consists in inoculating *Plasmodium* sporozoites while a treatment regimen is administered to which the parasite is susceptible. Chloroquine allows the development of the parasite in the liver, but kills the parasite's sexual forms when they are released into the bloodstream[98]. Pre-clinical evaluation has shown that this kind of immunization exposes the host to antigens from liver and blood stages of infection, and immunity against both stages was reported in a clinical trial[99]. Ten volunteers were exposed to *Pf* mosquito bite once a month for 3 months while receiving chloroquine prophylaxis. One month after the discontinuation of chloroquine, the volunteers were challenged with five mosquitoes of homologous *Pf*, and all 10 volunteers were protected[99]. Recent studies have shown that the CPS protocol didn't induce sterile protection against the erythrocytic *Pf* stages, demonstrated upon blood stage challenge. This strategy, although efficient, is difficult to implement due to prophylaxis routine ant the use of live and non-attenuated infected mosquitoes, but encourages a continued development of an effective preerythrocytic malaria vaccine[98].

### 1.3.3. *Pb*Vac vaccine

IMM's Prudêncio Lab proposed a new vaccination platform against human malaria, *Pb*Vac, in which the rodent parasite, *P. berghei*, expresses human *Pf* antigens as a safe, "naturally attenuated", whole-sporozoite (Wsp) vaccine. *Pf*CSP insertion into *Pb* is expected to generate humoral immunity against Pf, as well as cross species cellular immune responses, which may protect against a subsequent Pf infection. The candidate antigen was transfected to its delivery platform using 'gene insertion/marker out' (GIMO) methods of transfection.The gene encoding *Pf*CS was inserted into the neutral 230p locus of the *Pb* genome under the control of the 5′-and 3′ regulatory sequences of *Pb*'s uis4

gene (Fig. 2), which is expressed exclusively in sporozoites and developing liver stages. The GIMO transfection method employed ensures the stable insertion of the gene encoding the heterologous *Pf*CS and flanking regions in the *Pb* genome, resulting in a drug-selectable marker-free transgenic parasite. Genotyping of PbVac showed correct integration of the *Pf*CS expression"[100].

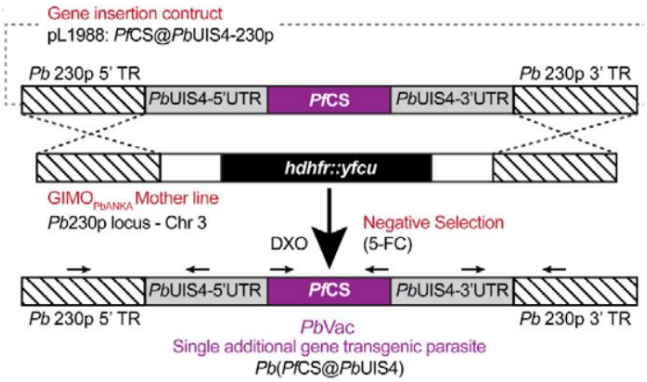


*Figure 2- Generation of PbVac. Schematic representation of the transgenic line PbVac line (Pb(PfCS@UIS4) where the GIMO 199 insertion-construct (pL1988) replaces the selectable marker (SM; hdhfr::yfcu) in the GIMO 200 PbANKA mother line with the PfCS coding sequence (CDS) after negative selection using 5- 201 fluorocytosine (5-FC). Construct pL1988 integrates by double cross-over homologous 202 recombination (DXO) using 5' and 3' targeting sequences (TR) for the neutral 230p locus, resulting 203 in the introduction of the PfCS CDS under the control of the PbUIS4 gene promoter (5'-UTR) and 204 Pbuis4 transcriptional terminator sequence (3'- UTR) and removal of the SM. Black arrows: 205 location of primers used for diagnostic PCR; 2018, Mendes M. A., A Plasmodium berghei Sporozoite-Based Vaccination Platform Against Human Malaria*

This genetically modified WSp vaccine can infect human hepatocytes but cannot continue to a blood stage infection, degenerating into unviable cryptic forms, which means that it may be non-pathogenic to humans. This evidence was further supported by studies in human hepatocytes cell lines, in liver and blood humanized mice and in New Zealand White (NZW) rabbits[100]. The *Pb*Vac parasite has been shown to display similar fitness to the wild type *Pb* parasite in terms of sporogonic development; formation of oocysts in the mosquito mid gut and of sporozoites in salivary glands; yield of exo-erythrocytic forms (EEFs); and hepatic load in infected mice and a higher human hepatocyte infectivity than *Pf* sporozoites in vivo[100].

Studies in NZW rabbits have shown that *Pb*Vac is not able to develop a blood stage infection, and that 10 days after administration the parasite has been eliminated from the liver and all the organs[101]. Notwithstanding this, as a safety measure, the capacity of Malarone® to eliminate hepatic *Pb*Vac parasites, and Malarone® and Coartem® to eliminate *Pb*Vac blood stage parasitemia from infected mice has been confirmed. In

addition, toxicological and humoral responses have been assessed in rabbits immunized 5 times via bite of 75 *Pb*Vac-infected *Anopheles stephensi* mosquitoes per administration. This study has shown that *Pb*Vac administration can trigger increased IgG titers anti-*Pf*CS and anti-*Pb*CS[101].

All these studies demonstrated the safety and immunogenicity of *Pb*Vac vaccine candidate, prompting its evaluation in non-human primates and subsequently in a Phase I/IIa clinical trial[101].

### 1.3.4. Immune response against vaccines

The complexity of the *Plasmodium* life cycle allows the conception of malaria vaccines that target different stages, and which may promote immunity by recognizing the parasite as a whole or of specific proteins. For blood-stage and transmission-blocking vaccine candidates immunity is mostly mediated by antibodies, whereas for pre-erythrocytic vaccines the type of immunity generated can vary, and involve both antibodies and CD4 T cells, such as in RTSs [102], as well as other T cells (CD8+, CD4+ and γδ T cells) such as in *Pf*SPZ[102].

Liver-stage vaccine candidates aim to prevent the progression of the parasite's life cycle to the blood stage through induction of potent liver-stage immune responses. This protective immunity is largely mediated by CD8+ T cells due to their robust parasite-induced responses and their capacity to eliminate the parasite. In rodents, CD8+ T cell responses were shown to be an efficient anti-parasite mechanism that eliminates malaria liver stages[103] and the depletion of IFN-γ or $CD8^+$ T cells blocked RAS-mediated sterile immunity[104].

Protection mechanisms behind RAS protection starts with antibodies that aim to inhibit sporozoites from reaching the liver and infecting hepatocytes, and the efficacy of this humoral response depends on the anti-sporozoite antibody titers[105]. Injected sporozoites when entering in the lymph nodes are presented by DC to prime specific CD8+ T cells that migrate to the liver and aim to eliminate the parasites and generate immunity[105]. CD4+ T cells can contribute positively to the proliferation and survival of CD8+ T cells, or with IFN-γ production, aiming to increase this response and its

durability[105]. Some of the circulating CD8+ memory T cells migrate to the liver, where they become tissue resident memory T cells[106]. CD8+ T cells surround these hepatocytes in order to eliminate them by IFN-γ production or through direct contact by cytolytic perforin and granzyme release[106]. Moreover, CD8+ T cells can induce RAS-protective immunity through the role of interleukins, nitric oxide (NO), and NK cells[106]. Iv *Pf*SPZ vaccination was shown to increase the levels of CD8+ T cells that produced effector cytokine molecules, such as IFN-γ, IL-2 and TNF-α, compared to pre-vaccination, as well as anti-CSP and anti-*Pf*SPZ antibody levels[92].

In GAS vaccine studies in mice, the protection conferred appears to depend on the time point at which the parasites arrest during liver-stage development[107]. Protective immunity was reported to be IFNγ-secreting CD8+ T cell-mediated, and long term protection was associated with CD8+ effector memory T cells. Protection studies in mice show that the immune response does not appear to be dependent on antibodies, and CD4+ T cell help is not essential to mount CD8+ T cell immune responses[107]. This immunization trigger antibody and B cell responses, but when B cells are depleted the mice were completely protected against a sporozoite challenge[108]. This study show that the immune response and protection are mainly mediated by CD8+ cells[108].

CPS immunization response is primarily mediated by parasite-specific antibodies that can interfere with sporozoite motility, hepatocyte invasion and development and by long-lasting-memory B cells. Besides that, cellular immune responses represent key effector mechanisms leading to parasite elimination[109]. Once again, IFN-γ CD8+ T cells elicit a crucial immune response through the increase in granzyme B, but Th1 are also important for this response as shown by the increase in transcription factors after CPS[110]. Partially and fully protective CPS volunteers show alterations in cytokine-producing γδ T cells by CD4+ and CD8+ effector memory T cells[111].

The immunity triggered by RTS,S is carried out by CSP-specific cell-mediated immunity complemented with CSP-specific antibody-mediated immunity. RTS,S cellular immune responses are essentially dependent on CD4+ T cells and IL-2 production, although other cytokines, including IFN-γ and TNF-α. have also been reported to be involved[112].

### 1.3.5. Rhesus monkeys as malaria vaccine trial models

The main animal model used in biomedical research in the world is the rodent model. However, although it has many advantages, it is not ideal for accurately mimicking the cellular and molecular mechanisms of the immune response and the response to infection in humans. Nonhuman primates (NHP), are able to bridge many shortcomings of other animal models, due to their significant genetic, physiological and behavioural homology, and are thus critical to understand pathogenesis, immunity and vaccine development in humans. However, some differences exist regarding markers of immune populations, namely NK, NKT cells and memory T cells[113].

Several studies evaluating malaria vaccines have been performed in rhesus monkey, such as studies on RTS,S vaccine adjuvants[114], IFNγ responses and antibody titers of RTS,S of adenovirus serotype-35 CSP prime and RTS,S boost[115]. $CD8^+$ T cell responses were correlated with protection against a challenge of *P. knowlesi* on rhesus inoculated with irradiated *P. knowlesi* sporozoites, which has been replicated in CHMI model with irradiated *Plasmodium falciparum* sporozoite vaccine[116]. NHP studies using *Pf*SPZ have shown that iv immunization triggers higher cellular immune responses than sc, especially with regard to *Pf*SPZ-specific IFN-γ, TNF-α and IL-2-producing CD8+ T cells. Analysis of the liver cells' immune responses 3 to 4 months after the last immunization showed that, while iv-immunized animals revealed a prevalence in magnitude of *Pf*SPZ-specific T cell responses, sc immunization resulted in low to undetectable T cell responses [117].

# 2. Aims

The asymptomatic and highly immunogenic nature of the *Plasmodium* liver stage makes it an ideal target for malaria vaccination development. IMM's MPrudêncio lab has developed *Pb*Vac, a new malaria vaccine candidate, in which the rodent parasite *P. berghei* expresses the human *P. falciparum* antigen *Pf*CSP in order to promote *Pf*CS-specific and cross-species immune responses that may protect against a subsequent *P. falciparum* infection. The pre-clinical phase of the vaccine candidate included experiments in mice, NZW rabbits and rhesus monkeys. Here we report the pre-clinical testing in rhesus monkeys of *P. berghei* WT and genetically modified sporozoites, as a strategy to induce immunity *to P. falciparum*.

The objectives established for this thesis are as follows:

- To confirm that the *P. berghei (Pb*) parasite infects rhesus hepatocytes *in vivo*.

- To analyse the humoral responses generated by specific immunization with *Pb*WT and *Pb*VAC sporozoites.

- To characterize the immune population dynamics in the liver, spleen and peripheral blood cells in response to immunization.

- To study the specific immune responses of PBMC and liver cells to *Pb*WT, *Pb*Vac and *Pf*.

# 3. Material and methods

All the experiments involving rhesus monkeys (*Macaca mulatta*)including immunizations, organ and blood collection, and cell isolation and processing were performed at the Biomedical Primate Research Centre (BPRC), Netherlands.

## 3.1. Animals

A total of 13 adult male rhesus macaques (*Macaca mulatta*) were infected at BPRC. One animal was used to confirm that *Pb*WT was capable of invading rhesus hepatocytes *in vivo*. Safety and immunogenicity were determined in twelve animals, randomised over three treatment groups: control (uninfected mosquitoes), *Pb*WT- and *Pb* (*Pf*CSP)@UIS4-infected mosquitoes (Table 1).

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*Table 1*- Characteristics of the animals used in the study. Weight and age mean, and respective standard deviations of the animals of each experimental group.

| Group | N | Weight | SD | Age (years) | SD |
|---|---|---|---|---|---|
| ***Infection test*** | 1 | 8,6 | - | 6,2 | - |
| ***Control*** | 4 | 10,2 | 1,6 | 8,2 | 3,9 |
| ***Pb*WT** | 4 | 10,4 | 1,4 | 6,8 | 1,3 |
| ***Pb* (*Pf*CSP) @ UIS4** | 4 | 10,2 | 1,7 | 7,1 | 1,0 |

## 3.2. Mosquito bite

Fifty mosquitoes were placed per cup and allowed to take a blood meal in the respective animal using an interrupted feeding schedule of 2, 3 and 10 minutes. For the *in vivo* confirmation, 150 mosquitoes infected with transgenic *P. berghei* parasites were used to infect each rhesus macaque. For immunisation assessments, 100 mosquitoes were used to immunize each animal with the respective parasite (see Table 1).

### 3.3. Study schedule

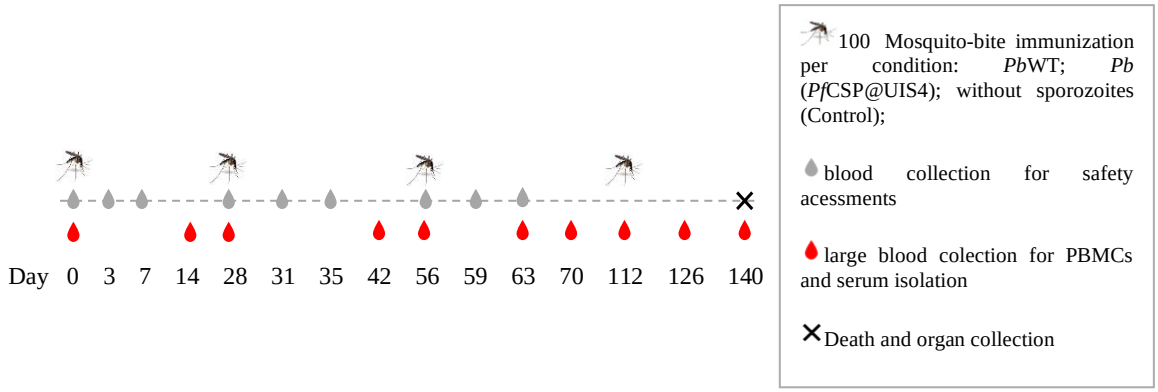


*Figure 3- Schedule of parasite administration, sample collection, and death in rhesus monkeys subjected to the bites of 100 non-infected, 4 with Pb WT and 4 with PbVac.*

Schedule of the experiment is illustrated in fugure 3. Immunizations were performed on days 0, 28, 56 and 112. On days 0, 3 and 7 after the first three immunizations, blood samples were collected, to anesthetized monkeys, to perform safety evaluations, to analyse haematological andclinical chemistry parameters and guarantee that the immunizations is safe and do not alter blood composition. On days 0, 14, 28, 42, 56, 63, 70, 112, 126 and 140 large bleeds were performed in order to access immunological parameters.

### 3.4. *In vivo* invasion

Following in vitro confirmation that *Pb* sporozoites invade rhesus hepatocytes, was performed a pilot experiment in one rhesus monkey to confirm liver infection in vivo. Two days after mosquito bite with *Pb* sporozoites, the animal was euthanized, the liver harvested and histological sections and staining were performed at BPRC. Liver schizonts were detected in liver slices by using Hoeschst to label DNA, as well as antibodies anti UIS4 or anti-heat shock protein 70 (HSP70) to label the parasite

### 3.5. Tissue collection and processing

After the animals were euthanized, livers were processed to extract and isolate viable mononuclear cells. Succinctly, the liver was perfused, the major vessels were clamped with a haemostat and cold phosphate-buffered saline (PBS) was used to elute the mononuclear cells.

After the liver was removed, it was placed in Roswell Park Memorial Institute 1640 (RPMI) (RPMI-1640 supplemented with 10% Pen-Strep-Glutamine and 10% heat inactivated fetal calf serum) (RPMI 10) on wet ice. The liver was perfused using cold PBS(1,5– 2 L) through the hepatic vein and collected through the portal vein in a 500mL centrifuge tube, and centrifuged (3000 rpm, 20 minutes, Temp 20°C). The supernatant was aspirated, and the cell suspensions were passed through -a 70 μm filter to a new 50mL vials to which 35 mL of PBS were added. 13mL of ficoll were added to the bottom of the cell suspension vials and centrifuged (2000 rpm, 30 minutes, Temp 20°C). The supernatant was discarded, and the buffy layer transferred to a new vial to which PBS was added and centrifuged (1200 rpm, 10 minutes, Accel 9, Brake 9, Temp 20°C), and this procedure was performed twice. Again, the supernatant was collected, but this resuspended in 25mL of pre-warmed RPMI10. The viable cells were counted and, centrifuged (1200 rpm, 10 minutes, Temp 20°C). The pellet was resuspended in freezing media (with a cellular concentration between 10 and 50 million cells per mL) and 1mL of suspension was pipette to cryovials to be immediately frozen.

The spleen was collected into RPMI10 medium on ice, cut into pieces of roughly 0.5$cm^3$, and transferred to nylon filters in 50mL conical vials where it was compressed using the end of the plunger from a 6 ml syringe. The filter was washed with PBS to allow cells to run through, and this procedure was repeated until entire the spleen was processed. Splenic suspension was re-filtered through a clean 70μm filter into a new 50mL vial. Then the same ficoll separation procedures were applied as for the liver, except that at the end the pellet was resuspended in 50 mL of pre-warmed RPMI10. Then the cells were equally counted, centrifuged, and resuspended at a cellular concentration of 10 to 50 million cells per ml in freezing media constituted by heat inactivated fetal calf serum with 10% of dimethyl sulfoxide (DMSO) and 1 ml of suspension were pipetted to cryovials to be frozen immediately. DMSO prevents that during the freezing process ice crystals are created that will induce cell death.

### 3.6. Collection and isolation of mononuclear cells

Blood samples from the large bleed collection (15-20 mL) were processed to extract viable mononuclear cells. This was performed in a Class II Biological Safety cabinet

using sterile technique. Whole blood was collected in acid-citrate-dextrose (ACD) or Ethylenediamine tetraacetic acid (EDTA) tubes and centrifuged (2500 rpm, 30 minutes, Temp 20°C). The plasma fraction was removed, and the remaining cellular suspension was diluted 1:1 with sterile PBS) Blood-PBS mixture was added to Ficoll and the same procedure as previously described for the organs was performed, followed by immediate cell freezing.

The cryopreserved cells were sent to the IMM, where they were kept at -80ºC.

### 3.7. Cell thawing

Samples were thawed at 37ºC in a water bath, shaking gently until only a small piece of ice was left. One ml of complete RPMI (cRPMI: RPMI 1640 (Gibco) supplemented with 1% (v/v) glutamine (Gibco), 1% (v/v) non-essential amino acids (Gibco), 1% (v/v) penicillin/streptomycin (Gibco), 10% (v/v) fetal bovine serum (FBS; Alfagene) and 1% (v/v) HEPES pH 7 (Alfagene)) was added drop by drop to the sample, which was then transferred, also drop by drop, to a falcon tube containing 8mL of cRPMI. The initial cell vial was washed with 1mL of cRPMI and cells were added to the falcon. Cells were centrifuged (1200 rpm, 10 min), the supernatant was discarded and the pellet was resuspended in 5 mL cRPMI. Cells were staied with 0,1% of Trypan Blue 25% (v/v) in PBS, a dye that stains dead cells blue, and counted on a Neubauer chamber.

Cells used for phenotyping were placed in the incubator at 37ºC and 5% C02 until the next day, when they were filtered using a 70µM filter, counted, centrifuged again and resuspended at a concentration of 1M/ 100µL in cRPMI. Cells used for intracellular cytokine assay were resuspended in cRPMI at a concentration of 1M/ 100µL and kept at 37ºC 1-2h until further use.

### 3.8. Sporozoites

For the quantification of antibody titers and for analysing the specific response were used *Pb*ANKA, *Pb*Vac and *Pf* NF54 sporozoites were obtained from dissection of the salivary glands of infected female *Anopheles stephensi* mosquitoes in non-supplemented

RPMI 1640 (Gibco) medium. Salivary glands were mechanically homogenized, filtered through a 40 μm strainer to a 50mL falcon to be centrifuged (1 minutes, 1200 RPM).

Up to 1mL of clear sporozoites was carefully added to 3 mL of 17% (v/v) Accudenz solution in MiliQ $H_2O$. Sporozoites were centrifuged (20minutes; 2500xg), and the top layer transferred to a 1,5 mL tubes and centrifuged again (15 minutes; 13000rpm; Temp. 4ºC). Supernatant was carefully removed and the pelleted sporozoites resuspended in a 10% (v/v) DMEM dilution to be counted in a in a Neubauer chamber using an OlympusCKX41 inverted microscope to be thawed in cryovials with $8*10^5$ sporozoites in each.

### 3.9. Quantification of antibody titers in the plasma by enzyme-linked immunosorbent assay (ELISA)

An ELISA was used to quantify total sporozoite-specific IgG in the plasma of immunized or non-immunized animals pre- (day 0) and post- (day 70) vaccination. This technique allows the detection of an antigen by the specific antibody linked to an enzyme, whose activity translates the amount of antibody bound, and once incubated with a substrate give rise to a measurable product[118].

A sporozoite extract was prepared by adding to 1M of cryopreserved sporozoites (*Pb*WT or *Pb*Vac) 100µL of extraction buffer (150mM NaCl; 20mM Tris-HCl; 1% triton; 1mM EDTA; Ph= 7,5) and 1µL of protease inhibitor and incubating 15 minutes on ice. The mixture was centrifuged (13000 rpm; 15 minutes), the supernatant diluted 1/160 in PBS and 50µL were used per well for coating the plates (ELISA MAX Uncoated plates-Biolegend), which were left overnight at 4ºC. On the next day, plates were washed with PBS and 150µL of blocking solution (5 % milk/ PBS + 0.05 % tween) was added for 1h at room temperature (RT). Post immunization samples were pooled to create a standard curve of 2-fold serial dilutions in 1% milk/ PBST (PBS 0.05% tween). All samples were diluted 1:50 in 1% milk/ PBST, and subsequently 1:2 and 1:4. The blocking solution was removed by washing 3x with PBST and 50µL of sample was added to the respective plate well. The plates were incubated for 3h at RT, washed 3 times with PBST and incubated with 100 µl of goat-anti-human IgG 1:1000 conjugated with horseradish peroxidase

(HRP) (Sigma - Aldrich) for 1h at RT. Plates were washed 3 times with PBST and 3 times with PBS and 100 µL of TMB (3,3',5,5'-Tetramethylbenzidine) (BD bioscience OptEIA), a substrate for HRP, were added to the well. The combination of HRP and hydrogen peroxide causes the oxidation of TMB and a colour change. The reaction was stopped by adding 50 uL of $H_2S0_4$, and the plate was read on a Tecan-M200 microplate reader at 450 nm.

To analyse total IgG titers against *Pb*WT or *Pb*Vac sporozoites, a standard curve was generated with serial dilutions of a mix of rhesus plasma from day 70. Absorbance from the control without plasma (background) was subtracted from all data points, and net absorbance was used to calculate antibody titers (in arbitrary units) by reporting to the line adjusted to the standard curve.

### 3.10. Quantification of plasma antibody titers by flow cytometry

Total sporozoite-specific IgG titers were further quantified by flow cytometry. This technique relies on the use of median fluorescence intensity (MFI) to determine the amount of antibody attached to a cellular antigen. Plasma samples from days 0 and 70 were tested against cryopreserved sporozoites, which were labelled with a red fluorescent cell-permeant nucleic acid stained SYTO61 dye (Invitrogen). Two types of controls were used, "SYTO only" without anti-IgG antibody, to guarantee the identification of the sporozoites only, and "no plasma" to ensure that there is no background and false positive IgGs.

Sporozoites were thawed in a 37ºC bath, 200µL of cRPMI were added and solution was transferred into 1,5mL tubes with 400µL cRPMI. Eppendorfs were centrifuged (15 minutes, maximum speed, Temp 4ºC), supernatant was carefully removed and sporozoites were diluted placed at a concentration of 150,000 sporozoites per 100µl cRPMI.

. Sporozoites were resuspended in 100µL of 20 µM SYTO 61, and placed in the fridge for 30 min. Sporozoites were then centrifuged (15 minutes, 13200 RPM, Temp. 4ºC), resuspended at $25x10^3$/20µL and 20ul added to each well of a 96 well plate. Plasma was diluted 1:500 in PBS, and 20 µL were added to the respective wells. Negative

controls (no SYTO61 labelling or no plasma) were also included. The plates were centrifuged (10 min., 3900 RPM, Temp. 4ºC), 20µL of anti-IgG CF488A (1:1000 in PBS; Sigma - Aldrich) were added to each well and incubated for 30 minutesat 4 ºC. Samples were fixed with 20µL of 2% formaldehydefor 10 minutesat 4 ºC, and then 200µL of cold PBS were added to pass to cytometry tubes. The samples were acquired in a BD LSR Fortessa X-20.

### 3.11. Optimization of flow cytometry panels

Flow cytometry is a single cell technique used to measure physical and chemical characteristics of a population of cells. A suspensions of cells is fluorescently labeled through expression of fluorescent proteins, staining with fluorescent dyes or with conjugated antibodies, and analyzed individually with a flow cytometer.

The fluidics system is responsible for the transport of the cells from the suspension tube to the flow cell through pressurized lines, ensuring individual cell alignment through the laser beam. The injection rate can be manipulated by the user.

The point where the cell interact with the laser is called interrogation point. The laser beam is emitted over the single cell and the scatter of the emitted light can be measured and correlated with some characteristics. Forward scatter (FSC) indicate the relative size of the cell and side scatter (SSC) indicates the internal complexity or granularity of the cell. The laser beam will also excite the fluorophores present in each single cell, causing it to emit fluorescent light that will be separated and targeted through a system of optical mirrors and filters according to the specific wavelengths of the respective optical detectors. After passing by the interrogation point, the fluidic system takes the cell to the waste container.

The emitted light detected is converted to voltage when amplified and signal obtained becomes digital in BD FACSDiva$^{TM}$ software.

Fluorophores attached to the antibodies absorbing in a range of specific wavelengths (absorption spectrum) result in excitement of electrons, which emit light in a higher wavelength (emission spectrum) when returning to their ground state of energy. The different absortion and emission sectra of fluorophores allow their separation using

optical filters. The fluorescent compounds added to a cell can identify different cell populations, surface receptors, intracellular organelles, immunophenotyping, measure enzyme activity and apoptotic cell populations and determine nucleic acid content. The most widely fluorochromes used are fluorescein isothiocyanate (FITC), phycoerythrin (PE) and allophycocyanin eF780 (APCeF780). They have also been developed in response to the increased number of colors detected by cytometers tandem dyes containing two fluorochromes, as such as PE-Cy5 and APC-Cy7 for example. When the first die reaches the maximal absorbance, the energy is transferred to the second dye that will emit fluorescence. When multiple fluorochromes are used, an overlap of the emission spectra of the fluorochromes can occur, that is called spectral overlap. Colour compensation is the technique used to correct this overlap, trough the subtraction of a fraction of the signal from the fluorochrome that is spilling over into that channel. To know how much compensation is needed single-colour control samples should be run, to measure the fluorochrome signal detected in other channels, and apply compensation to remove the signal.

BD LSRFortessa X-20 is a multicolor analysis with four lasers violet (405 nm), blue (488 nm), yellow-green (561 nm) and red (640 nm), and each one has different detectors with filters for a set of fluorochromes.

This complex technique requires a long process of optimizations. The construction of the panels need to consider several aspects, such as the maximum number of parameters that BD LRSFortessa X-20 can analyse, the limited colours of antibodies reactive with rhesus available and the conjugation of the fluorochromes. Therefore, in order to create antibody panels to analyze the populations of interest, we included both rhesus-reactive anti-human antibodies after appropriate testing, as well as specific anti-rhesus antibodies. Once the panels were decided and created according to the criteria described, the antibodies were titrated to identify the ideal concentration at which it is possible to distinguish between the negative and the positive populations using concentrations of 1:80, 1:160 and 1:320. After testing these standard concentrations it was necessary for some of the antibodies to test other dilutions. And finally the candidate panels were tested to ensure that the conjugation of the antibodies in their respective fluorochromes and in the decided concentrations worked (tables 2 and 3). This entire

optimization process was performed with rhesus monkey PBMCs and the compensations were performed with compensating beads, used as single-color controls.

2 panels were created to incorporate all populations of interest, panel 1 (table 2) focused on identifying antigen presenting cells, ILCs, NK and NKT cells and TCRγδ cells, and panel 2 to identify T and B lymphocytes and the respective memory and regulatory/helper cells(table 3). All panels included a fixable viability dye (FVD) to label dead cells.

*Table 2*- Panel 1 to analyse the phenotype of the cells. Panel focused on identifying NKT cells, TCRγδ cells, NK, dendritic cells, monocytes and ILCs

| Fluorochrome | Panel 2 | Clone | Company | Dilution factor |
|---|---|---|---|---|
| BV785 | CD127 | A019D5 | BioLegend | 1:40 |
| BV711 | BDCA-1 | L161 | BioLegend | 1:640 |
| BV650 | HLA-DR | L243 | BioLegend | 1:160 |
| BV605 | CD16 | 3G8 | BioLegend | 1:80 |
| BV510 | CD3 | V500 | | 1:40 |
| BV421 | CD11c | 301627 | | 1:160 |
| PerCPCy5.5 | CD4 | OKT4 | BioLegend | 1:80 |
| FITC | CD21 | B-ly4 | BioLegend | 1:40 |
| PE-Cy7 | CD123 | 6H6 | | 1:200 |
| PE-Cy5 | CD20 | 2H7 | BioLegend | 1:320 |
| PE-Dazzle594 | CD14 | M5E2 | BioLegend | 1:640 |
| PE | TCRγδ | B1 | BioLegend | 1:160 |
| APCeF780 | FVD | - | eBioscience | 1:80 |
| AF700 | CD8 | RPA-T8 | BioLegend | 1:40 |
| AF647 | CD45 | MB4-6D6 | Miltenyi Biotec | 1:80 |

*Table 3*- Panel 2 to analyse the phenotype of the cells. Panel focused on identifying T and B lymphocytes, and respective memory cells.

| Fluorochrome | Panel 1 | Clone | Company | Dilution factor |
|---|---|---|---|---|
| BV785 | CD127 | A019D5 | BioLegend | 1:40 |
| BV711 | CCR7 | G043H7 | BioLegend | 1:80 |
| BV650 | CD69 | FN50 | | 1:80 |
| BV605 | CD40L | 24-31 | BioLegend | 1:80 |
| BV510 | CD3 | V500 | | 1:40 |
| BV421 | CD27 | | | 1:200 |
| PerCPCy5.5 | CD4 | OKT4 | BioLegend | 1:80 |
| FITC | CD21 | B-ly4 | BioLegend | 1:40 |
| PE-Cy7 | CD25 | 2A3 | BD Bioscience | 1:40 |
| PE-Cy5 | CD20 | 2H7 | BioLegend | 1:320 |
| PE-Dazzle594 | CD14 | M5E2 | BioLegend | 1:640 |
| PE | CXCR5 | MU5UBEE | eBioscience | 1:640 |
| APCeF780 | FVD | - | eBioscience | 1:80 |
| AF700 | CD8 | RPA-T8 | BioLegend | 1:40 |
| AF647 | CD95 | DX2 | BioLegend | 1:320 |

To quantify the specific immune responses by intracellular cytokine assay (ICS), another panel was used (table 4), in which some antibodies were stained at the surface and others intracellularly according to the method described later.

*Table 4*- Panel to analyse specific responses of the cells against stimulus. (*) Antibodies used as intracellular staining; the rest were used for surface staining.

| Fluorochrome | Stimulation | Clone | Company | Dilution factor |
|---|---|---|---|---|
| BV711 | CCR7 | G043H7 | BioLegend | 1:80 |
| BV650 | CD69* | FN50 | | 1:80 |
| BV605 | CD16 | 3G8 | BioLegend | 1:120 |
| BV510 | CD3* | V500 | | 1:40 |
| BV421 | TNF-α* | Mab11 502929 | | 1:80 |
| PerCPCy5.5 | CD4* | OKT4 | BioLegend | 1:80 |
| FITC | IL-2* | MQ1-17H12 | | 1:40 |
| PE-Dazzle594 | IFNγ* | 4S.B3 502545 | | 1:160 |
| PE | TCRγδ | B1 | BioLegend | 1:160 |
| APCeF780 | FVD | - | eBioscience | 1:80 |
| AF700 | CD8* | RPA-T8 | BioLegend | 1:40 |
| AF647 | CD95 | DX2 | BioLegend | 1:320 |

Data analysis consists in the identification of cells or population of interest through a method denominated gating, that select the populations of interest. The gating strategy can be visualized in a histogram, that represent the number of events in the y-axis for a parameters signal value in channel numbers in x-axis, or in a 2-D graph, a two-parameter analysis. The analysis of the acquired data was performed with FlowJo V10.

### 3.12. Phenotypic characterization of immune populations in the blood, liver and spleen of immunized vs. non immunized monkeys

PBMCs were analysed before the first immunization (day 0) and on day 140. Liver mononuclear cells and splenocytes were analysed on day 140.

Cells were thawed and placed at a concentration of $1x10^7$ cells /ml in PBS2 (PBS with 2% of serum). One million cells (100µL) were placed per well in a 96 U-bottom well plate and centrifuged (5 minutes, 1600rpm), and supernatant was removed by flipping the plate. 20µL of FVD eFluor™ 780 from eBioscience were added for 15 min. at RT, and then 100µL of PBS2 were added per well and the plate was centrifuged (5minutes, 1600rpm). Cells were surface-stained using with the antibodies described on tables 3 and

4 at the respective concentrations and were incubated 20 minutes at RT. Cells were washed with 100µl of PBS2, centrifuged (5 minutes, 1600rpm) and were fixed in 100µL of formaldehyde 1% for 5 minutes at RT. Cells were washed with 100µL of PBS2, were centrifuged (5 minutes, 1600rpm), and were added 200µl of PBS2 per well totransfer to FACS tubes. About 500,000 events per tube were aquired on a BD Fortessa X-20 whenever possible and the analysis was performed with FlowJo V10.

Due to the lack of sample or enough cells to be analysed, results on PBMCs and liver cells are missing for some immunized monkeys.

### 3.13. Quantification of specific immune responses by ICS

Specific immune responses of PBMCs and liver mononuclear cells against sporozoites were analysed at day 140 through intracellular cytokine assay. Briefly, cells were stimulated with cryopreserved *Pb*WT, *Pb*Vac or *Pf* sporozoites and cytokine production was measured by flow cytometry. A non-stimulated control and a positive control were always included per monkey, and brefeldin was added to all samples in the final 5h. The phenotype and the stimulation assays of each monkey were performed on the same day.

Cells and sporozoites were thawed as described above. Cells were distributed in U-bottom 96 wells plate, at a concentration of $1x10^7$ cells /ml in cRPMI per well/condition. 100µL of stimuli were added to each well. To non-stimulated wells 100µL of cRPMI were added, and to the positive control, 80µL of cRPMI, and plates were placed during 17 hours at 37ºC and 5% $CO_2$. The positive control is a combination of phorbol myristate acetate (PMA; final concentration 10 ng/mL) with ionomycin (final concentration 500 ng/mL) to induce the maximum intracellular capacity to produce cytokines. 20µL of this solution were added per well to each positive control. One hour later 20µL of brefeldin (final concentration 10µg/mL) were added to all the wells to inhibit the protein transport from the golgi complex to the endoplasmic reticulum keeping them inside the cell, and the cells were incubated for 5h at 37ºC and 5% $CO_2$.

After the last incubation, the plates were centrifuged (5 min., 1600rpm), 20µL of FVD 1:3200 were added and cells were 15 minutes at RT. Cells were washed with 100µL

of PBS2/ well, centrifuged (5 min., 1600rpm) and 20µL of a surface antibody mix (with the antibodies at the appropriate dilutions) in PBS2 was added. Stained cells were incubated 30 minutes at RT, 100µL of PBS were added, plate was centrifuged and 100µL of Fix/Perm (fixation/permeabilization concentrate diluted 1:4 in fixation/permeabilization diluent; Invitrogen) was added, and the cells were further incubated in the fridge for 30 minutes. Next the plates were centrifuged and cells were washed with permeabilization buffer diluted 10x in water. Fix/Perm and permeabilization buffer are a kit used to fix cells in suspension and permeabilize the membrane of the cells in order to facilitate the intracellular antibodies access to intracellular structures and leaves the morphological light-scattering characteristics of the cells intact at the same time as reduce background staining. Antibodies for intracellular staining were added (20µL of a mix of antibodies diluted in permeabilization buffer to the appropriate conentrations), and were incubated in the fridge for 30 minutes. The cells were washed with 100µL of PBS2, centrifuged (5 min., 1600rpm), and 200µL of PBS2 were added each well. Cells were transferred to cytometer tubes and an additional 100µL of PBS2 were added. All wash steps aim to reduce the background of the cells, allowing population distinction. About 500,000 events were acquired on BD Fortessa X-20 and the analysis was performed using FlowJo V10.

Due to the lack of sample or enough cells to be analysed, results on PBMCs and liver cells are missing for some immunized monkeys.

### 3.14. Statistical analysis

Due to the small number of samples per group it is not possible to test the normality of the variables and therefore non-parametric statistical tests were used. To compare between two groups in which both samples consist in distinct test subjects a non-paired Mann-Whitney statistical test was used, and for paired samples Wilcoxon matched-pairs signed rank tests were applied[119]. Differences were considered statistically significant when P-values were lower than 0.05, and represented in the figures as: *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$.

To analyze correlations nonparametric Spearman correlation was used. A correlation result = -1 means an inverse correlation, = 0 that the two variables do not vary at all together and = 1 a perfect correlation. A small p-value reject the idea that the correlation is due to random sampling.

Statistical analysis and all the graphs were performed in GraphPad Prism 7.

# 4. Results

## 4.1. Invasion of rhesus hepatocytes by *Pb*WT *in vivo*

We first asked whether *Pb*WT was able to infect rhesus hepatocytes *in vivo*, since this had not been previously addressed and was crucial for the validation of the model. One monkey was inoculated with *Pb*WT sporozoites and 2 days later infection was confirmed in histological slides of liver sections (Fig.4). This validated the use of *Pb*WT for the immunization of rhesus macaques.

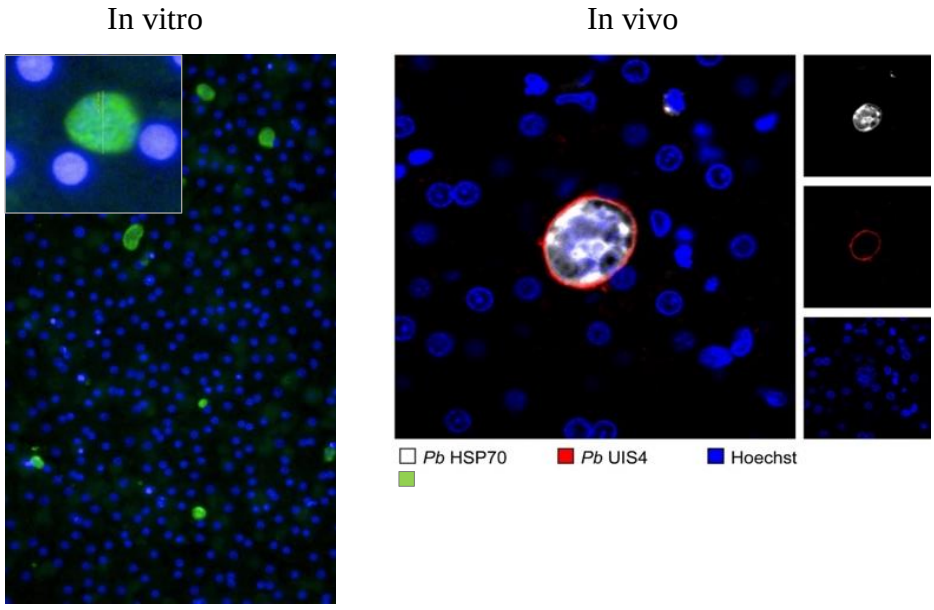


*Figure 4- Rhesus hepatocytes infection with PbWT sporozoites. In vitro infection of primary rhesus hepatocytes with PbWT parasites, and histological liver slices of PbWT infecting rhesus hepatocytes in vivo.*

## 4.2. Safety evaluations

According to the study scedule shown above, several parameters were determined by haematology and clinical chemistry throughout the study on freshly obtained blood samples at the BPRC. The following biochemical evaluations were performed: alkaline phosphatase, alanine transaminase (ALAT), aspartate transaminase (ASAT), gamma glutamyl transpeptidase (γ-GT), bilirubin, cholesterol, lactate dehydrogenase (LDH), urea, creatinine, albumin, total protein, glucose, iron, calcium, sodium, potassium,

chloride, phosphate and bicarbonate (HCO3-). The following haematological evaluations were performed: erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume (MVC), mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, white blood cell count including leukocyte differentiation, platelet count and mean platelet volume. All these data were compared with reference values obtained from healthy naïve rhesus monkeys, and clinical parameters that were altered during the course of the study were due to frequent sedation and blood collection, and as such were consistent between control and immunized monkeys.

The animals were also evaluated for their weight and behaviour, that remained constant.

To confirm that there were no breakthroughs thick smears and PCR were performed. No breakthroughs were detected, with parasite not being found in the blood.

In general, the mosquito-delivered *Pb*WT and *Pb*Vac sporozoite inoculations were well tolerated and no severe adverse events were observed.

### 4.3. Humoral immune responses

The humoral immune responses of rhesus macaques immunized with *Pb*WT or *Pb*Vac were determined in plasma samples by ELISA (Fig. 5). Total IgG titers against *Pb*WT or *Pb*Vac sporozoites were calculated relatively to a standard curve, and presented as the difference between post (day 70) and pre (day 0) immunization samples.

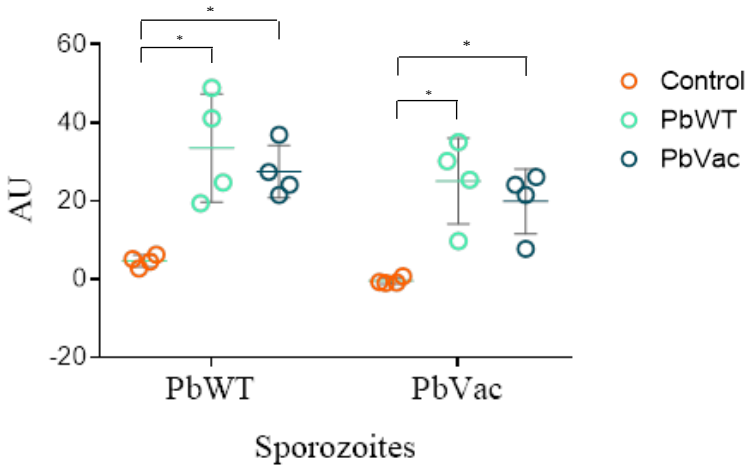


*Figure 5- IgG titers were determined in rhesus plasma by ELISA (difference between day 70 and day 0). IgG concentrations are presented as arbitrary units, calculated from a standard curve made from a pool of rhesus plasma at day 70. To verify significance between day 0 and day 70 of the plasma of each monkey were used paired Wilcoxon tests and Mann-Whitney statistical between the animals groups.*

Immunized animals presented higher IgG titers than control monkeys, confirming the generation of a humoral immune response against *Pb*WT and *Pb*Vac. In addition, we found no significant differences in sporozoite-specific IgG titers between *Pb*WT- and *Pb*Vac-immunized animals.

Sporozoite-specific IgG titers were further tested in a modified ELISA assay where whole sporozoites were used as antigen and quantification was performed by flow cytometry. *Pf* sporozoites were analysed in parallel with *Pb*WT and *Pb*Vac in order to evaluate the specific humoral response. Median fluorescence intensity (MFI) was used to determine relative IgG titers, and the results are presented as the difference between post (day 70) and pre (day 0) immunization samples (Fig. 6).

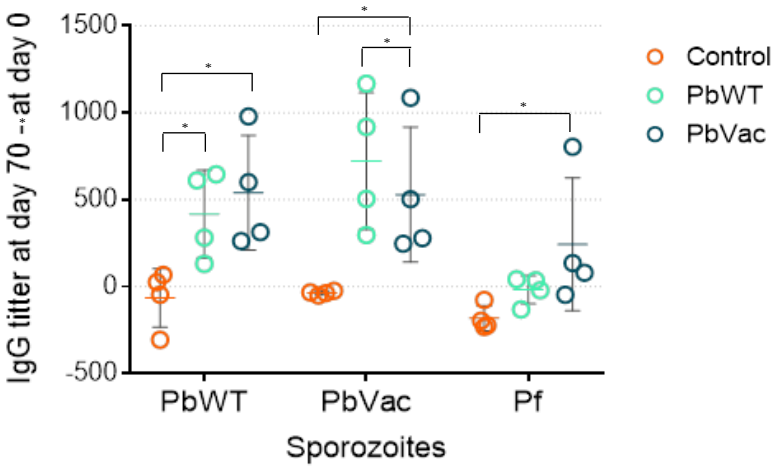


*Figure 6- IgG titers were determined in rhesus plasma by Flow cytometry (difference between day 70 and day 0). IgG concentrations are presented as arbitrary units, calculated from a standard curve made from a pool of rhesus plasma at day 70. To verify significance between day 0 and day 70 of the plasma of each monkey were used paired Wilcoxon tests and Mann-Whitney statistical between the animals groups.*

Total IgG titers against *Pb*WT and *Pb*Vac sporozoites increased significantly with immunization, in agreement with the ELISA results. Of note, no statistical significant differences were observed between *Pb*WT- and *Pb*Vac-immunized monkeys. These results further confirm the generation of a humoral response to both *Pb*Vac and *Pb*WT.

Importantly, we also found increased IgG titers against *Pf* sporozoites in both *Pb*WT- and *Pb*Vac-immunized as compared to non-immunized monkeys, although this was only significant for the later. This supports the generation of an anti-*Pf* humoral response elicited by *Pb*Vac immunization and indicates that the *Pf*CS protein present on *Pb*Vac but not *Pb*WT may be involved in this response.

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## 4.4. Phenotypic characterization of immune populations in the blood, liver and spleen of immunized vs. non immunized monkeys

Next we characterized the landscape of immune populations described to be involved in response to infection or vaccination in the blood and tissues of immunized and non-immunized macaques by flow cytometry. The populations analysed and the corresponding markers used to identify each population are presented in Fig. 7.

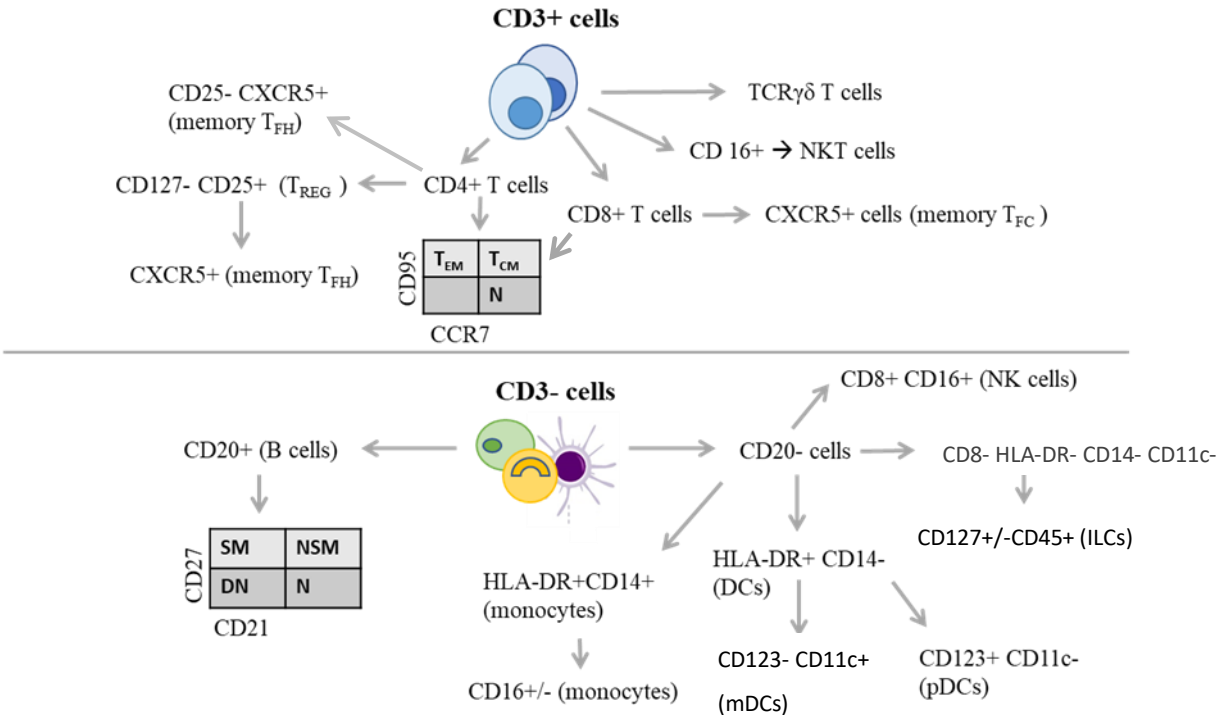


*Figure 7- Phenotypic populations to be analysed by flow cytometry.*

We first analysed the frequency of the most important innate populations in the blood in order to compare the alterations in cell populations with immunization. The populations analysed included monocytes (CD14+HLA-DR+), myeloid DCs (HLA-DR+CD14-CD11c+CD123-), plasmacytoid DCs (HLA-DR+CD14-CD11c-CD123)+, NK (CD16+/-CD8+), NKT (CD16+) and ILCs (Lin-CD45+D127+/-). The gating strategy used to identify all the populations is presented in supplementary figure 1 (annex 1) and data is presented as fold change between day 140 and day 0 (Fig. 8).

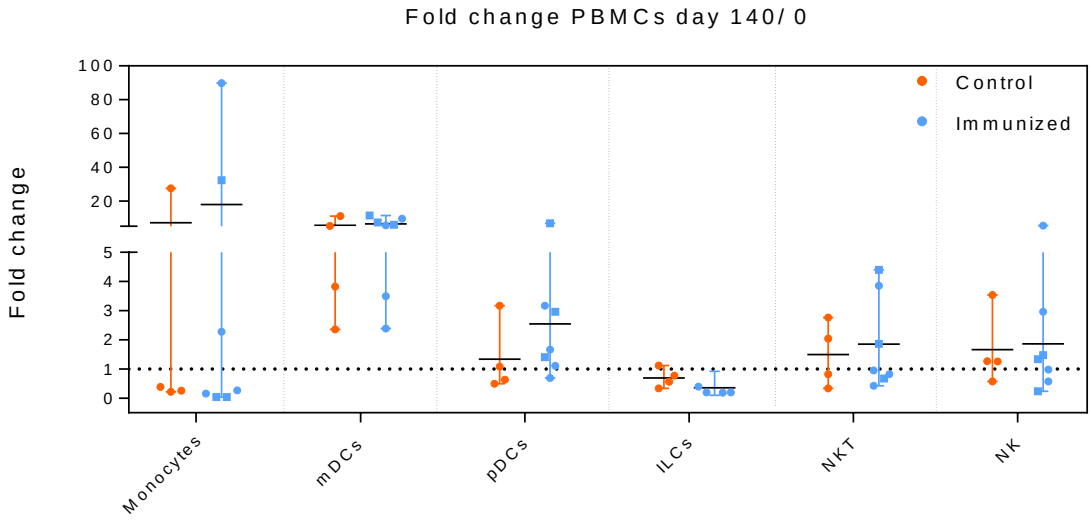


*Figure 8- Alterations in innate populations with immunization.*. Innate populations were analysed within PBMCs at days 0 and 140 according to the gating strategy presented on annex 2. Statistical significance was assessed usiang Mann-Whitney non-parametric tests. ■ *PbWT immunization;* ● *PbVac immunization*

Although none of the modifications from control to immunized animals were significant, there was a slight decrease in ILCs on day 140 compared with day 0 and an increase in plasmacytoid DCs.

The same immune populations were then analysed in tissue (liver and spleen), and PBMCs from the last day of the experiment (Fig. 9).

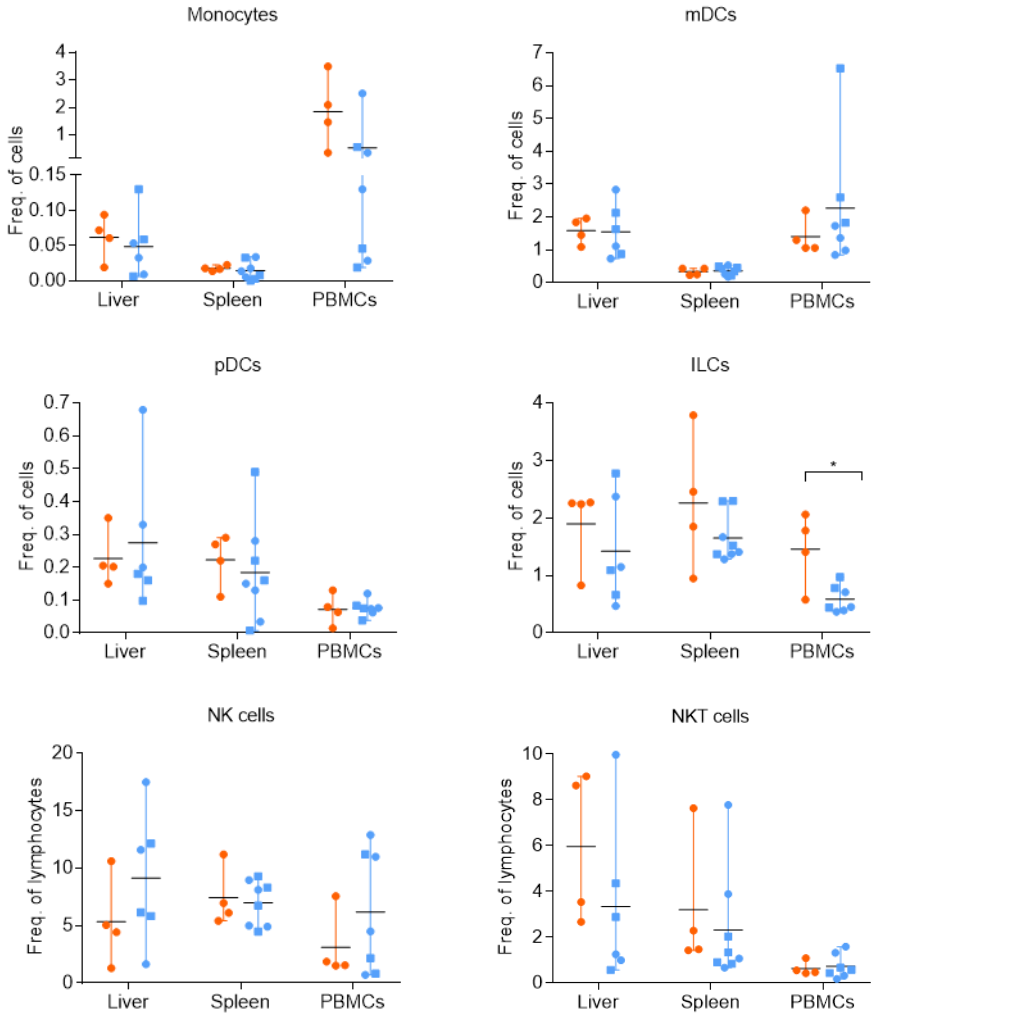


*Figure 9- Frequency of monocytes, DCs, ILCs, NK and NKT cells in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 1 and is presented as frequency within total leukocytes. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ *PbWT immunization;* ● *PbVac immunization*

Monocytes were found to be more present in PBMCs than in tissues, as expected. In addition, we found a decrease in monocyte frequency in the blood but not in the liver or spleen in immunized animals, although this was not significant. Frequency of dendritic cells did not show significant differences. NK cells were increased in liver cells and

PBMCs of immunized animals, although this was not significant. NKT lymphocytes were decreased in the the spleen of immunized animals, although not significanlly.

Interestingly, we found a decrease in ILC frequency in all organs in immunized as compared to non-immunized animals, and this decrease was statistically significant in PBMCs.

Next we analysed the impact of immunization on the circulating T and B cell compartments by comparing the fold change from day 0 to day 140 of immunized and non-immunized groups (Fig. 10). An extensive analysis of the following T cell populations was performed: total T cells (CD3+), TCRαß T cells (TCRαß+; CD16-), CD4 T cells (CD4+CD8-); CD4 Tfh cells (CXCR5+; CD25-); CD4 Treg cells (CD25+; CD127-); CD4 Tfr cells (CXCR5+); CD8 T cells (CD8+CD4-); CD8 Tfc (CXCR5+) and TCRγδ T cells (TCRαß; CD16+), and also B cells (CD20+; CD3-). The gating strategy is presented in annex 1 and 2.

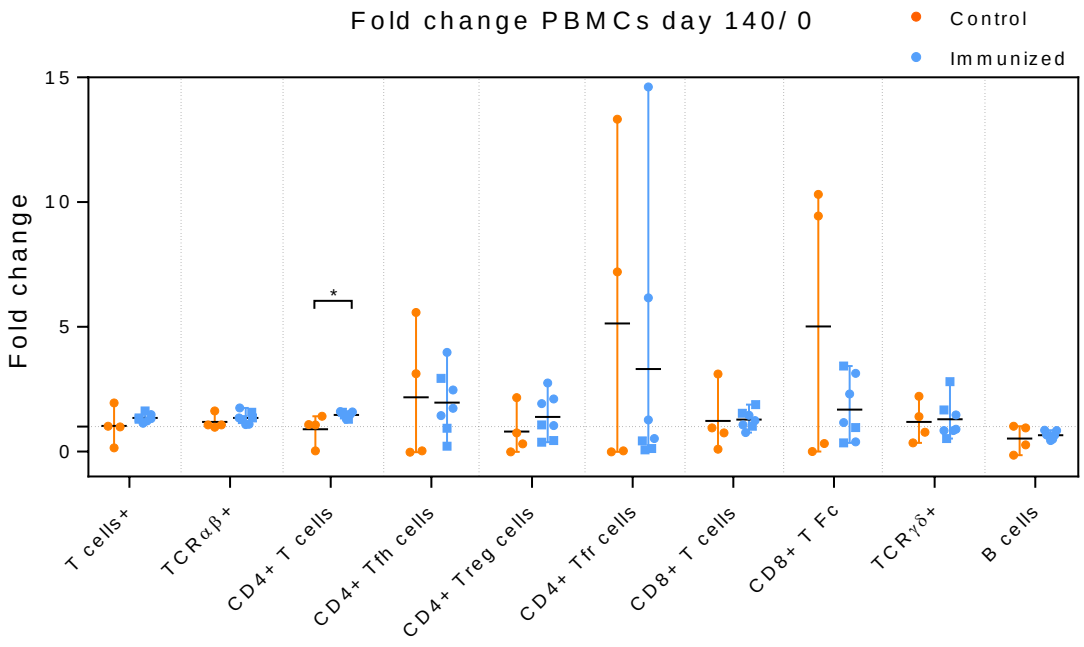


*Figure 10- Alterations of the T cell compartment and B cells within total PBMCs of immunized and non-immunized monkeys from days 0 to 140, frequency within total lymphocytes. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ *PbWT immunization;* ● *PbVac immunization*

Overall we found that the circulating T cell compartment was very robust and did not present major alterations with immunization. Importantly, we found a mild but significant increase in CD4+ T cells in the immunized group as compared to the control group.

Next we compared the frequency of all T cell subsets in the liver and spleen as well as PBMCs in immunized vs. non-immunized animals.

First we analysed total T cells as well as the TCRαβ and TCRγδ subsets. We found no overall alterations in total T cell frequency in the spleen or PBMCs with immunization, but a clear decrease (although not significant) in total T cells in the liver (Fig. 11). When we analysed TCRαβ and TCRγδ T cells separately we could conclude that the decrease observed was due to lower frequency of the later subset in the liver. In addition, we found that TCRγδ T cells were significantly decreased in the spleens of immunized monkeys, in contrast to the non significant complementary increase in TCRαβ T cells in the same organ.

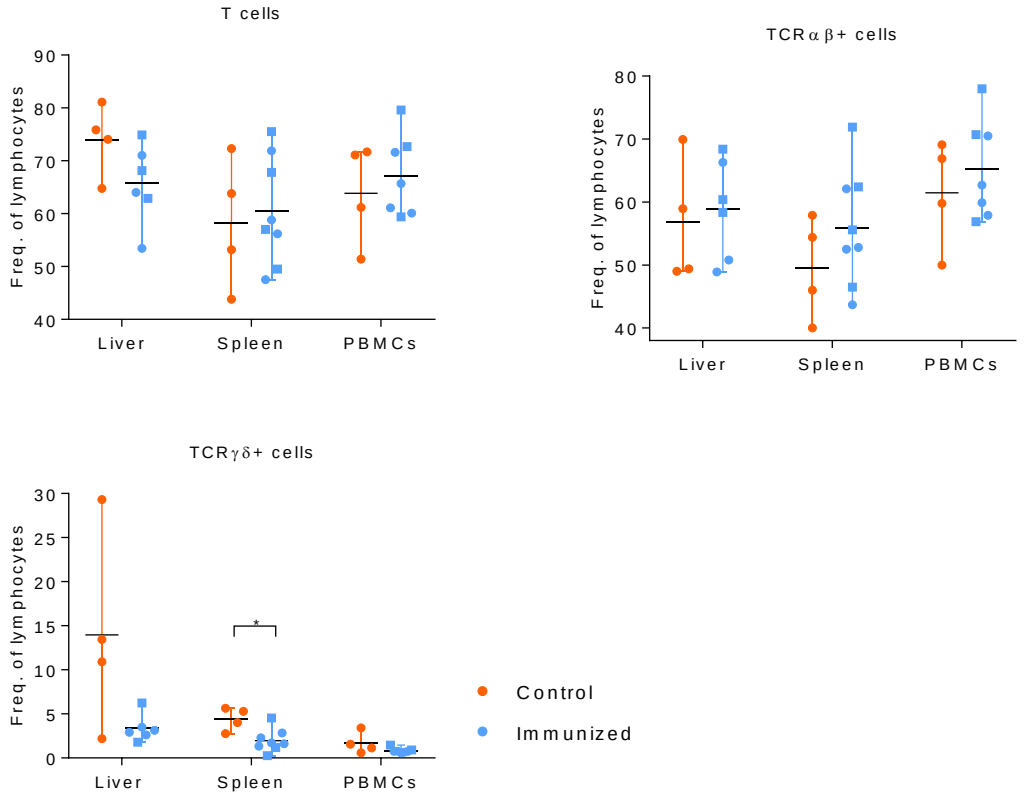


*Figure 11- Frequency of T cells, and the 2 major TCR recepors in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 1 and is presented as frequency within total lymphocytes. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ *PbWT immunization;* ● *PbVac immunization.*

The frequency of CD4+ T cells was found to be higher in PBMCs than in liver and spleen (Fig. 12A), In addition, CD4+ T cell frequency increased after immunization both in PBMCs, in line with the results presented in Fig. 10, and in the liver, although this was not significant. There were no alterations in the frequency of naïve and memory CD4+ T cells with immunization in the spleen or in PBMCs (Fig. 12B). Nevertheless, we observed an increase in the naïve compartment in the liver, which was mirrored by a decrease in memory CD4+ T cells (Fig. 12B). We further found an increase in liver Treg cells, which have been reported to increase in infectious contexts, in immunized animals, although this was not significant. Finally, Tfh cells showed a slight, non significant increase in immunized monkeys in both tissues and blood, while Tfr cells presented a trend for increased frequency upon immunization in both tissues.

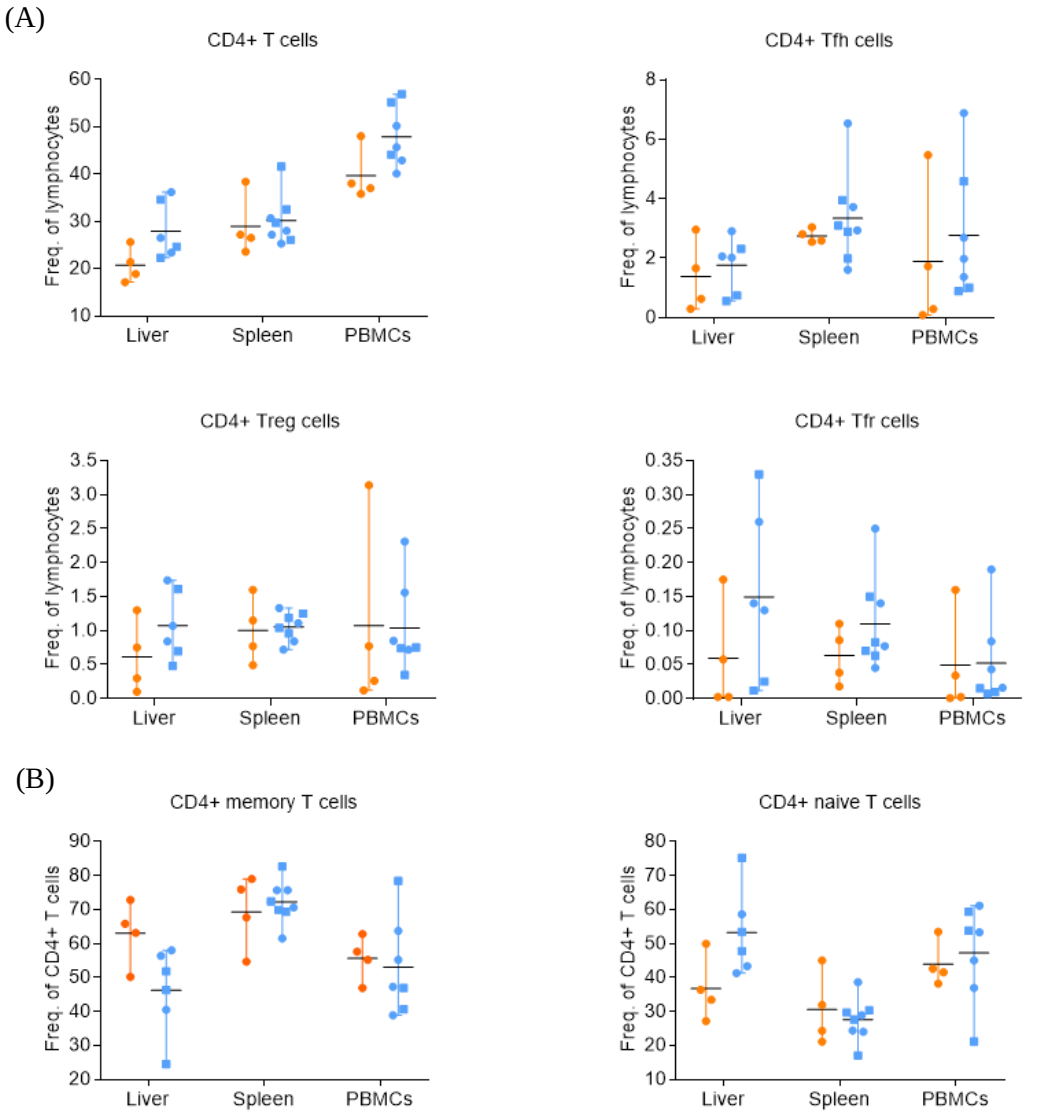


*Figure 12- Frequency of CD4 cell subsets in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 2 and is presented as frequency within (A) total lymphocytes, and within (B) CD4+ cells in memory analysis. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ *PbWT immunization;* ● *PbVac immunization.*

Surprisingly, we found lower statistically significant frequency of CD8+ T cells in the liver after immunization (Fig. 13). This is unexpected since this cell subset has been reported to play a key role in protection against pre-erythrocytic stages of malaria infection[104]. Regarding naïve and memory CD8+ T cell subsets, an increase in the later was found only in the spleen upon immunization, in parallel with a decrease in the former

subset. Finally, follicular cytotoxic CD8+ cells did not reveal major differences between the two groups of animals.

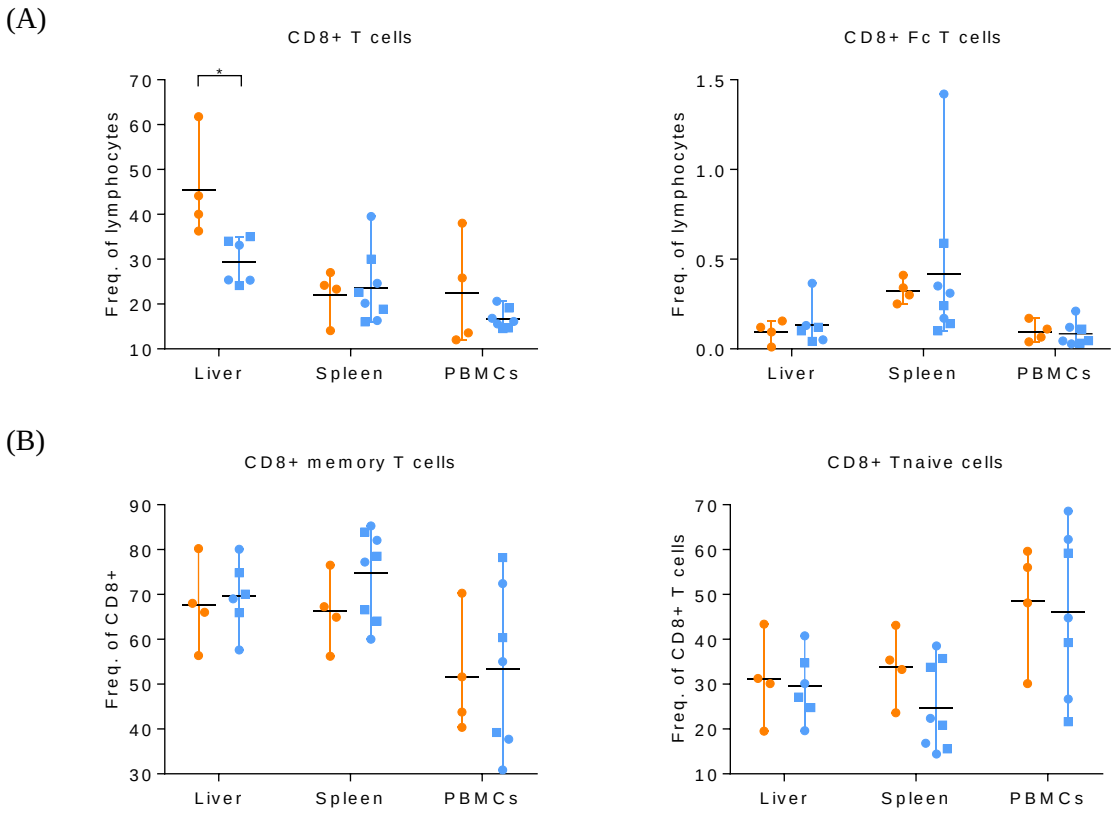


*Figure 13- Frequency of* CD8 cell subsets *in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 2 and is presented as frequency within (A) total lymphocytes, and within (B) CD8+ cells in memory analysis.* Statistical significance was assessed usiang Mann-Whitney non-parametric tests. ■ PbWT immunization; ● PbVac immunization.

$T_{RM}$, identified by CD69 expression, showed a statistically significant decrease in the liver of immunized animals, which is due to a decrease in CD8+ but not CD4+ T cells (Fig. 14). As expected, CD4+ $T_{RM}$ cells represented a much lower frequency of total lymphocytes in the liver than CD8+ $T_{RM}$ cells. Finally, CD4+ $T_{RM}$ cells presented increased frequencies in the spleen of immunized monkeys, although this was not statistically significant.

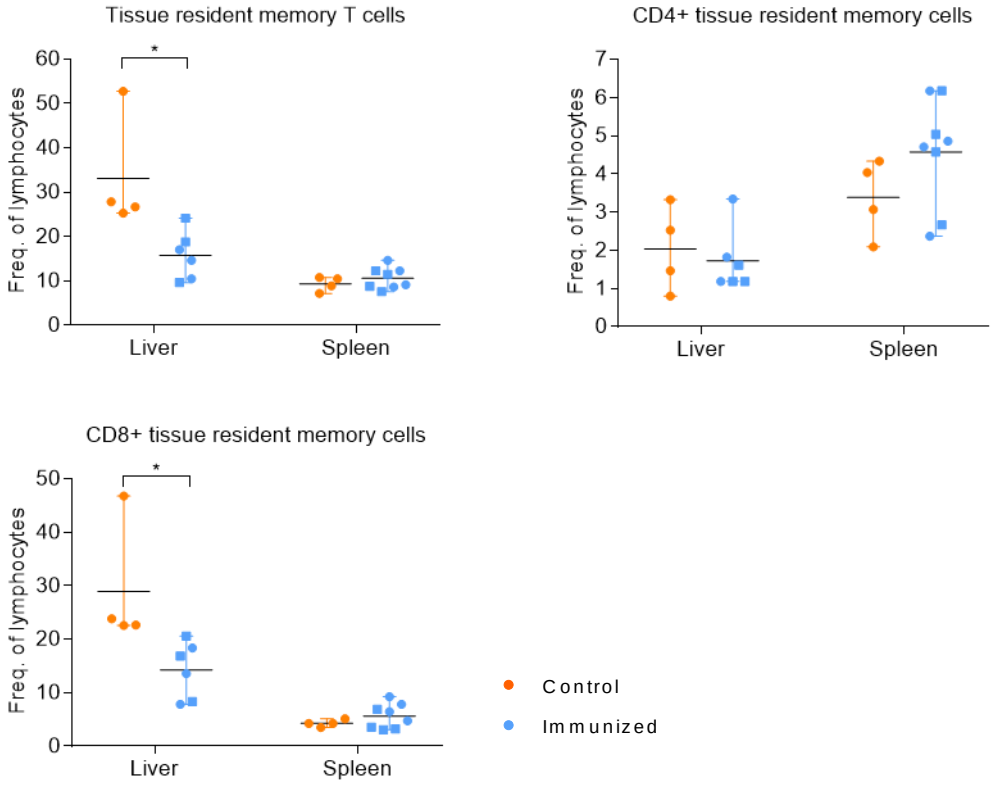


*Figure 14- Frequency of* tissue resident memory cells in *tissues on day 140 post-immunization. Data was analysed as depicted in annex 2 and is presented as frequency within total lymphocytes.* Statistical significance was assessed usiang Mann-Whitney non-parametric tests. ■ *PbWT immunization;* ● *PbVac immunization.*

Total B cells showed only a slight increase in the liver of immunized animals (Fig. 15).

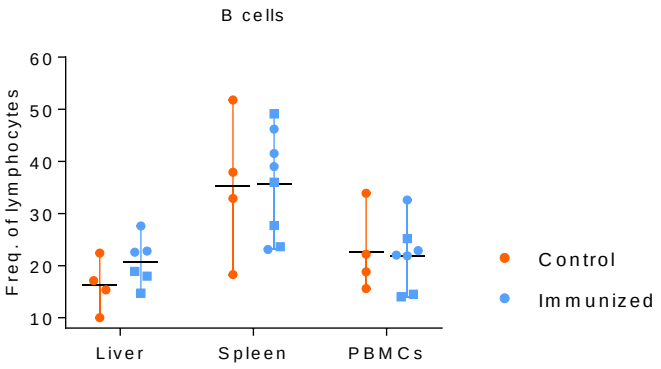


*Figure 15- Frequency of total B cells in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 2 and is presented as frequency within total lymphocytes. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ PbWT immunization; ● PbVac immunization.

Finally, analysis of the naïve and memory B cell subsets revealed no statistically significant changes associated with immunization (Fig. 16). Nevertheless, a trend for increased unswitched memory B cell frequencies was found in the spleen of immunized animals.

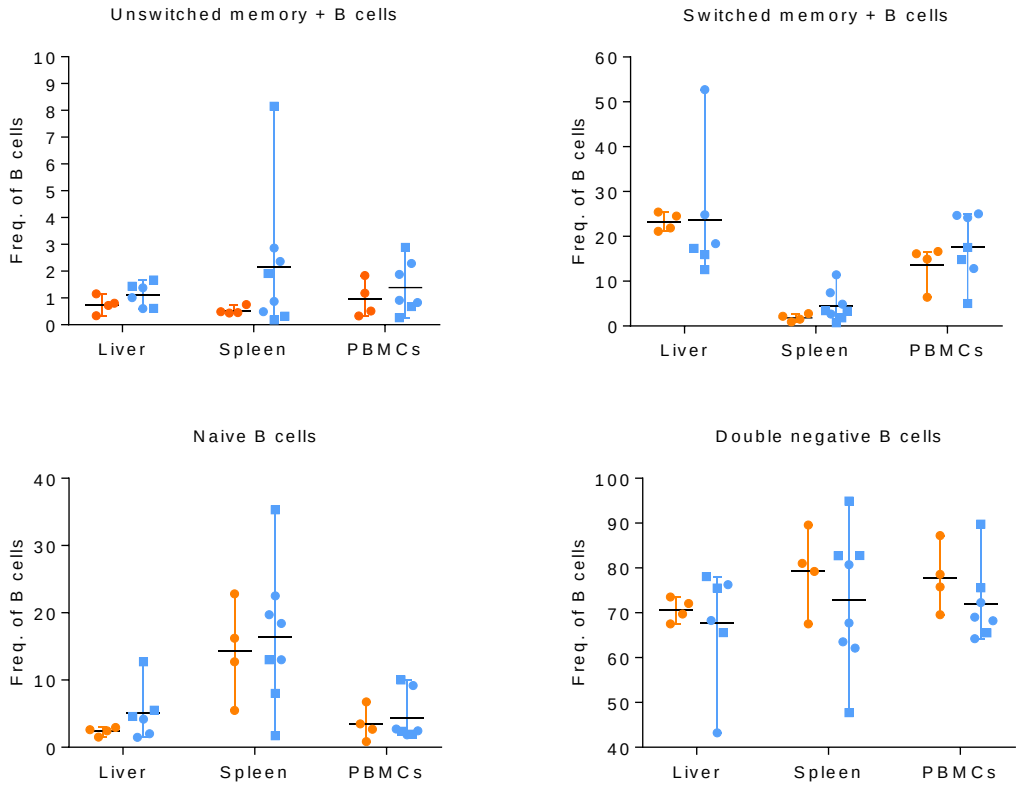


*Figure 16- Frequency of B cell subsets in blood and tissues on day 140 post-immunization. Data was analysed as depicted in annex 2 and is presented as frequency within total lymphocytes. Statistical significance was assessed usiang Mann-Whitney non-parametric tests.* ■ *PbWT immunization;* ● *PbVac immunization.*

## 4.5. Correlations between blood and tissue populations

A major advantage of this work is the ability to compare the circulating compartment, that is usually assessed in human studies, with tissue-specific alterations. We thus analysed the correlations between the frequencies of immune populations in blood cells and tissues, and also between tissues (Table 5).

The analyse of the correlation between the frequencies of populations between blood cells and tissues, and also between tissues are presented in table 6. The p-values

that give consistency to the results of r, showing that increase/ decrease in both tissues is common, are marked in blue. Thus we can see that the increase of NK in PBMCs and liver is consistent, as well as the decrease of NKT in spleen with the other two samples. As well as the frequency of the Tfh population between the PBMCs and the liver and all Treg correlations tend to increase together. And finally, in memory B cells there is a slight increase between PBMCs and the spleen and a decrease in them in double negative (DN) cells.

*Table 5- Analysis of cell frequency correlation between blood cells and tissues and between both tissues. Correlation was assessed usiang Spearmen-r tests with 95% interval confidence, correlation coefficient, r, ranges from -1, an inverse correlation to +1, a perfect correlation. The p-values that give consistency to the results of r, showing that increase / decrease in both tissues is common, are marked in blue.*

| | **PBMCs- Liver** | | **PBMCs- Spleen** | | **Liver- Spleen** | |
|---|---|---|---|---|---|---|
| | Spearman- r | p-value | Spearman- r | p-value | Spearman- r | p-value |
| **Monocytes** | 0,2845 | 0,454 | 0,2586 | 0,4379 | 0,3842 | 0,2722 |
| **mDCs** | 0,06695 | 0,8683 | -0,3904 | 0,2324 | -0,07903 | 0,8302 |
| **pDCs** | 0,2167 | 0,5809 | -0,3189 | 0,3364 | 0,006079 | 0,992 |
| **ILCs** | -0,1333 | 0,7435 | 0,2545 | 0,4511 | 0,4255 | 0,2203 |
| **NK cells** | 0,7333 | 0,0311 (*) | 0,2636 | 0,4348 | 0,7697 | 0,0126 (*) |
| **NKT cells** | 0,65 | 0,0666 | 0,8455 | 0,0018 (**) | 0,697 | 0,0306 (*) |
| **T cells** | 0,4 | 0,2912 | 0,2273 | 0,5034 | 0,103 | 0,785 |
| **TCRαß** | 0 | >0,9999 | 0,06364 | 0,8603 | 0,5152 | 0,1334 |
| **TCRγδ** | 0,5799 | 0,1076 | 0,5114 | 0,1107 | 0,103 | 0,785 |
| **CD4 T cells** | 0,4667 | 0,2125 | 0,3827 | 0,2441 | 0,4681 | 0,1738 |
| **CD4 Tfh cells** | 0,9667 | 0,0002 (***) | -0,1727 | 0,6147 | -0,2242 | 0,5367 |
| **CD4 Treg cells** | 0,7833 | 0,0172 (*) | 0,6909 | 0,0226 (*) | 0,7333 | 0,0202 (*) |
| **CD4 Tfr cells** | 0,6 | 0,0968 | 0,2727 | 0,4181 | -0,006061 | >0,9999 |
| **CD4 memory cells** | 0,1088 | 0,7844 | 0,222 | 0,5073 | 0,3891 | 0,2659 |
| **CD4 naive cells** | 0,15 | 0,7081 | 0,2091 | 0,5393 | 0,2606 | 0,4697 |
| **CD8 T cells** | 0,3833 | 0,3125 | 0,3182 | 0,3415 | -0,0303 | 0,946 |
| **CD8 Fc cells** | 0,6778 | 0,0514 | 0,09112 | 0,7905 | 0,04863 | 0,899 |
| **CD8 memory cells** | 0,3667 | 0,3363 | 0,2 | 0,5574 | 0,2727 | 0,4483 |
| **CD8 naive cells** | 0,2667 | 0,4933 | 0,2455 | 0,4684 | 0,2727 | 0,4483 |
| **TRM cells** | - | - | - | - | -0,05455 | 0,8916 |
| **CD4 TRM cells** | - | - | - | - | -0,1043 | 0,7822 |
| **CD8 TRM cells** | - | - | - | - | -0,09091 | 0,8113 |
| **B cells** | 0,35 | 0,3586 | 0,2364 | 0,4854 | 0,6485 | 0,049 (*) |
| **NSM B cells** | 0,4333 | 0,2499 | 0,9021 | 0,0003 (***) | 0,2067 | 0,5652 |
| **SM B cells** | 0,2667 | 0,4933 | 0,4364 | 0,1826 | 0,1636 | 0,6567 |
| **Naive B cells** | 0,1833 | 0,6436 | 0,1913 | 0,5703 | -0,1824 | 0,6136 |
| **DN B cells** | 0,2833 | 0,463 | 0,6364 | 0,0402 (*) | -0,2364 | 0,5135 |

We found a positive correlation between NK cells in the liver and in the blood or the spleen, indicating that liver NK frequency can be accurately assessed systemically. CD4 Tfh and CD4 Treg cells were the only other populations that were positively correlated between liver and PBMCs, and thus may represent accurate tissue information from the blood. Interestingly, CD4 Treg frequency was always highly correlated amongst all compartments analysed, indicating systemic regulation. And finally, in memory B cells there is a consistent result between PBMCs and spleen. Even so, there are not many correlations between changes in blood and tissues, which means that regulation of cellular changes occurs locally in each tissue, and when we look at blood prefile it is not possible to interpolate to tissues.

## 4.2. Cellular immune responses

We analyzed blood and liver cells of immunized and non immunized animals for the production of the Th1-associated cytokines IL-2, IFN-γ and TNF-α upon antigen (sporozoite) encounter using an ICS assay. Cells were stimulated with *Pb*WT or *Pb*Vac sporozoites, as well as with *Pf* sporozoites, during 22 hours and the intracellular specific production of cytokines by different populations was analysed. IFN-γ aims to increase immune response and its durability[105], TNF-α is involved in controlling parasite burden and activate other effector cells such as NK cells and macrophages[64] and IL-2 is a cytokine associated with memory T cells and T cell proliferation and differentiation In the case of specific stimulation it is relevant to distinguish between *Pb*WT and *Pb*Vac immunized monkeys, so results are presented as separate groups in the graphs.

Analysis of total lymphocytes revealed low responses to all types of sporozoites in immunized monkeys, which were indistinguishable from the responses of non-immunizaed controls (Fig. 17). Nevertheless, cytokine production was higher in liver cells than in PBMCs, specially against *Pb*Vac.

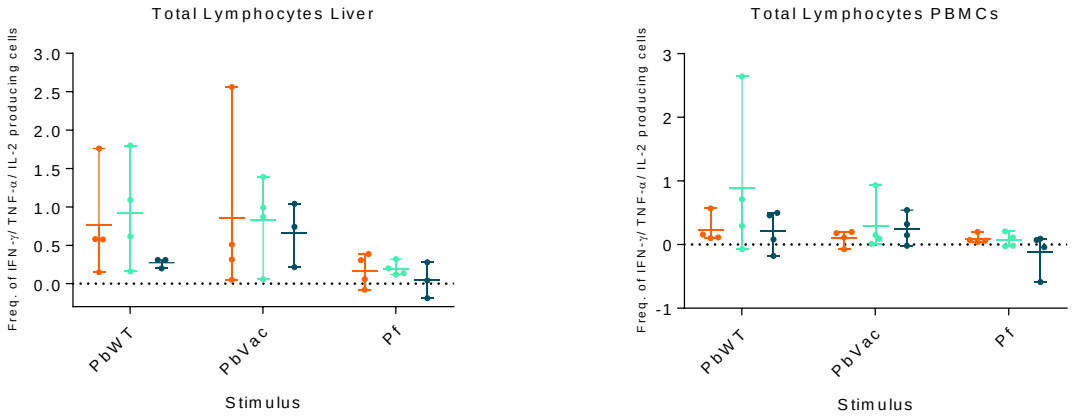


Figure 17- Analysis of total lymphocytes specific response in liver cells and PBMCs in liver and PBMCs. Cytokine production is presented as a result of the production of any of the three cytokine (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gatting strategy used it was the same as to analyze the phenotype. Statistical significance was assessed usiang Mann-Whitney non-parametric tests

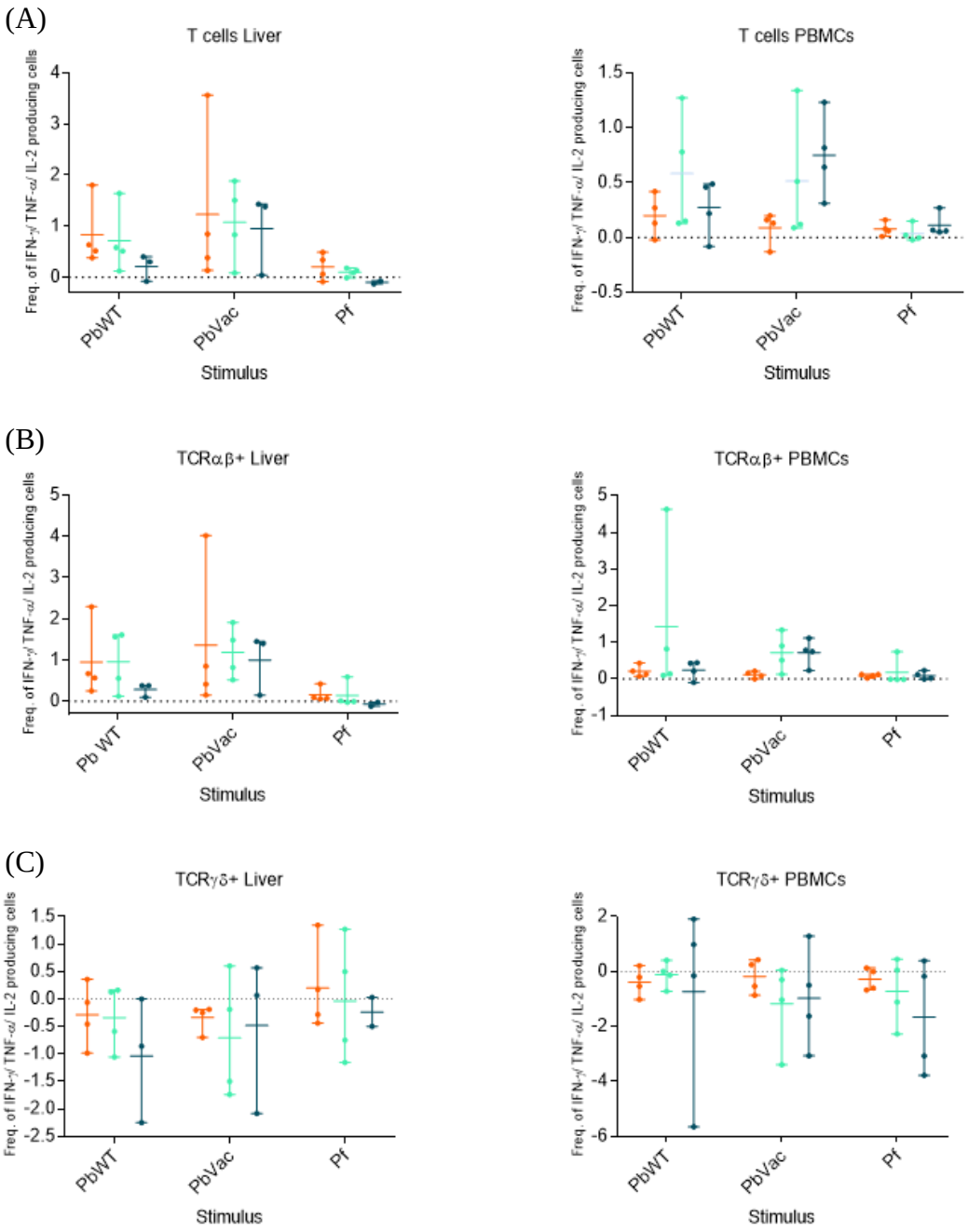


*Figure 18- Analysis specific responses of T cells (A) and the major T cell subsets (B) in liver and PBMCs. Cytokine production is presented as a result of the production of any of the three cytokines (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gating strategy used was the same as to analyze the phenotype. Statistical significance was assessed usiang Mann-Whitney non-parametric tests*

Within total T cells (Fig. 18A), higher specific responses in immunized vs non.immunized monkeys were only found in the blood in response to *Pb*Vac stimulation, although this was not significant. This trend was most probably due to αß T cell and not

γδ T cell cytokine production (Fig. 18 B, C). Regarding γδ T cells, their specific responses to all stimulus in both blood and liver were negligible .

We next analysed the specific responses of CD4+ T cells (Fig. 19A). Liver cells from *Pb*WT-immunized animals showed a trend for increased cytokine production upon stimulation with any of the sporozoites relative to control monkeys, and this was not observed in *Pb*Vac-immunized animals. In contrast, increased specific responses in the blood of immunized animals were only observed upon stimulation with *Pb*Vac sporozoites, and these were statistically significant in *Pb*WT-immunized monkeys. Similar results were obtained when analysing memory populations, although statistical significance was lost for the later case (Fig. 19B).

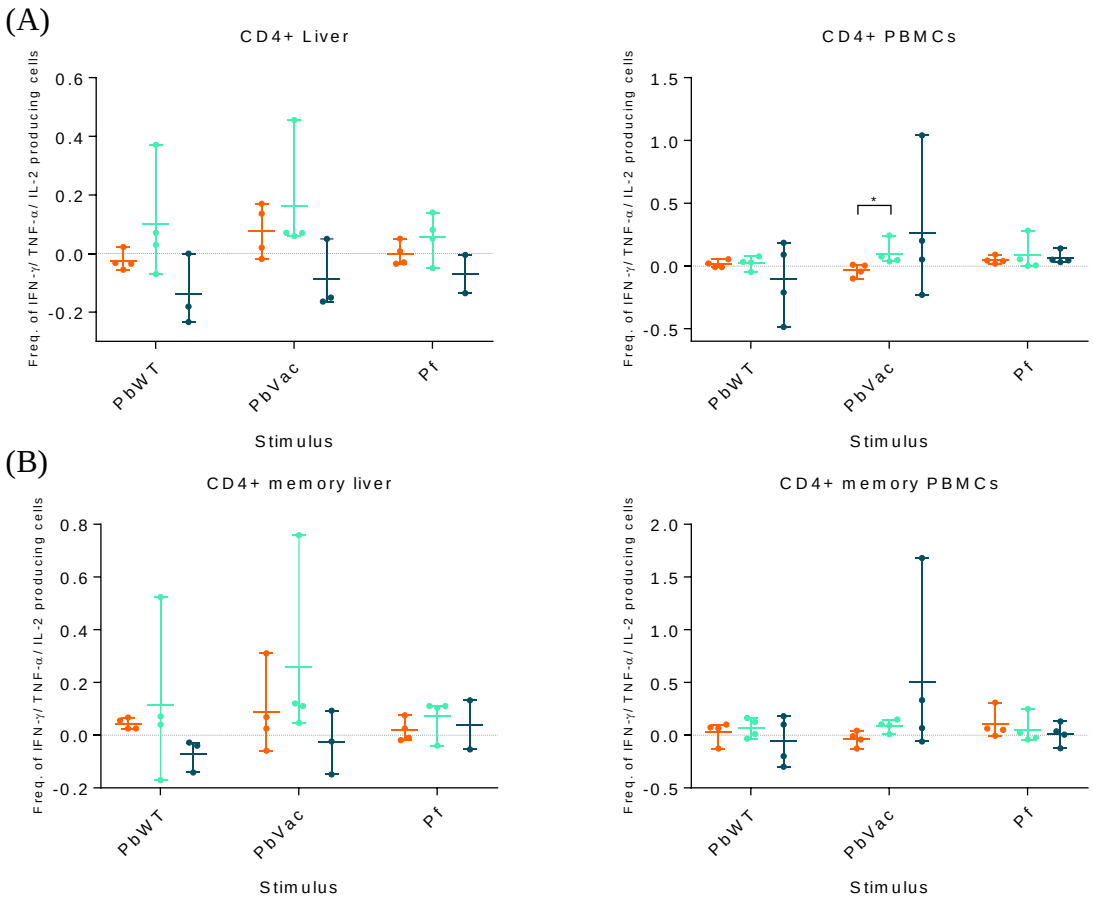


Figure 19- Analysis of CD4+ T cells (A) and memory cells (B) specific response in liver and PBMCs. Cytokine production is presented as a result of the production of any of the three cytokine (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gatting strategy used it was the same as to analyze the phenotype. Statistical significance was assessed usiang Mann-Whitney non-parametric tests

Cytokine production by total CD4+ T cells was further dissected in terms of mono- and poly-functional cells (Supplementary figure 3). The results in the liver show that the responsible for the increase in cytokine production of *Pb*WT animals stimulated by *Pb*WT is TNF-α. Regarding *Pb*Vac stimulation, only a slight increase occurs in TNF-α in both immunized animals and an increase of IFN-γ in *Pb*Vac immunized monkeys. *Pf* stimulation increased the frequency of TNF-α in *Pb*WT monkeys and IL-2 on *Pb*Vac monkeys. On PBMCs, PbWT stimulation induce a decrease of TNF-α in both immunized monkeys and an increase of IL-2 in *Pb*Vac-immunized monkeys, *Pb*Vac stimulation lead to an increase of IL-2 in immunized animals that translate into an increase in the total frequency. Also *Pf* stimulation show an increase of IL-2 in *Pb*Vac monkeys. Although very inconsistent, and not statistically significant, we can say that the most often repeated variable is an increase in TNF-α in the liver and IL-2 in PBMCs.

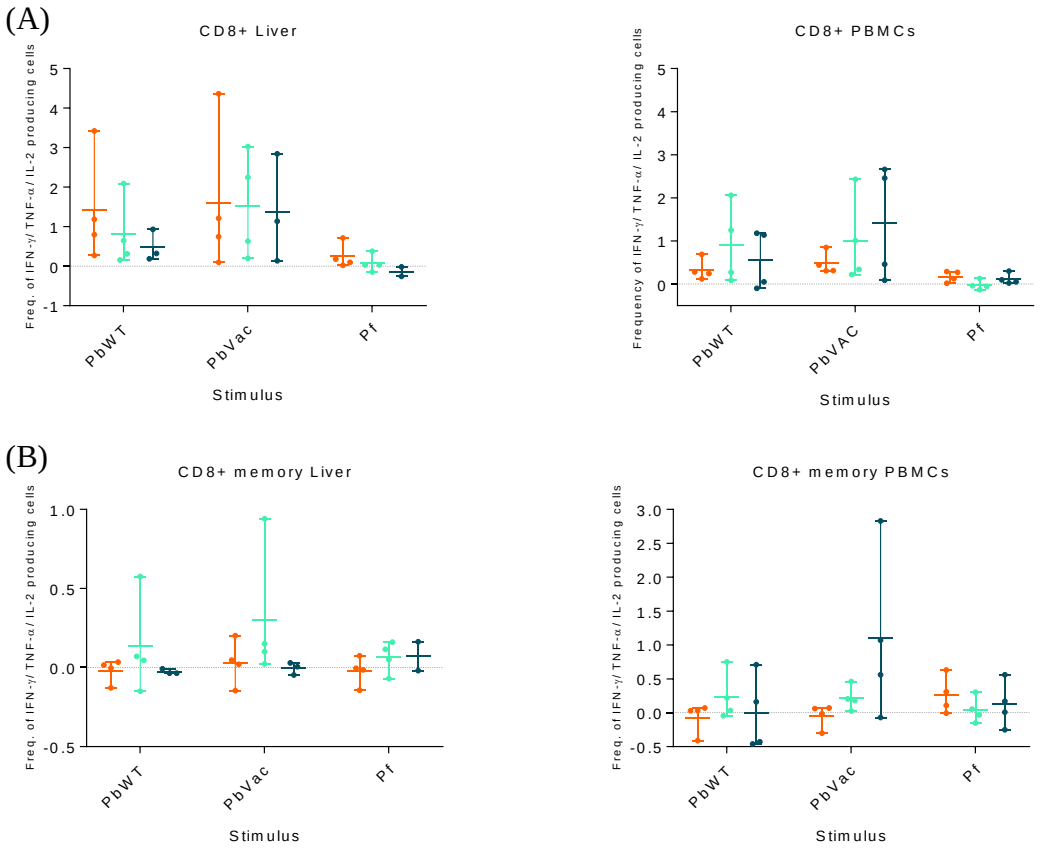


*Figure 20*- Analysis of specific responses to CD8+ T cells (A) and CD8+ memory T cells (B) in liver and PBMCs. Cytokine production *is presented as a result of the production of any of the three cytokine (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gatting strategy used it was the same as to analyze the phenotype.* Statistical significance was assessed usiang Mann-Whitney non-parametric tests

We then analysed specific CD8+ T cell responses in the liver and blood of immunized vs. non-immunized animals. Regarding blood cells, *Pb*WT and *Pb*Vac, but not *Pf* stimulation, induced an increase on cytokine production in immunized animals. Within the CD8+ memory T cell population this trend was maintained, although *Pb*WT-immunized animals showed only a slight increase in response *Pb*Vac stimuli. In the liver an increased response was observed in *Pb*WT-immunized animals when stimulated with *Pb*WT or *Pb*Vac, but this was not statis+tically significant. We further analysed mono- and poly-functional cells within total CD8+ T cells in response to sporozoite stimulation, but found no relevant results (Supplementary figure 4).

In conclusion, none of the T cell subsets from immunized animals analysed showed increased cytokine production upon stimulation with *Pf* sporozoites, indicating that *Pb*Vac immunization may not elicit cross-species cellular responses in rhess monkeys.

The potential of NKT cells reside in the rapid release of cytokines that promote or suppress different immune responses. Both liver and blood NKT cell responses were limited and not different between immunized and non-immunized controls (Fig. 21A). This was also observed for NK cells (Fig. 21B), except for the response of *Pb*Vac-immunized animals against Pf, which was significantly decreased upon stimulation relatively to the control.

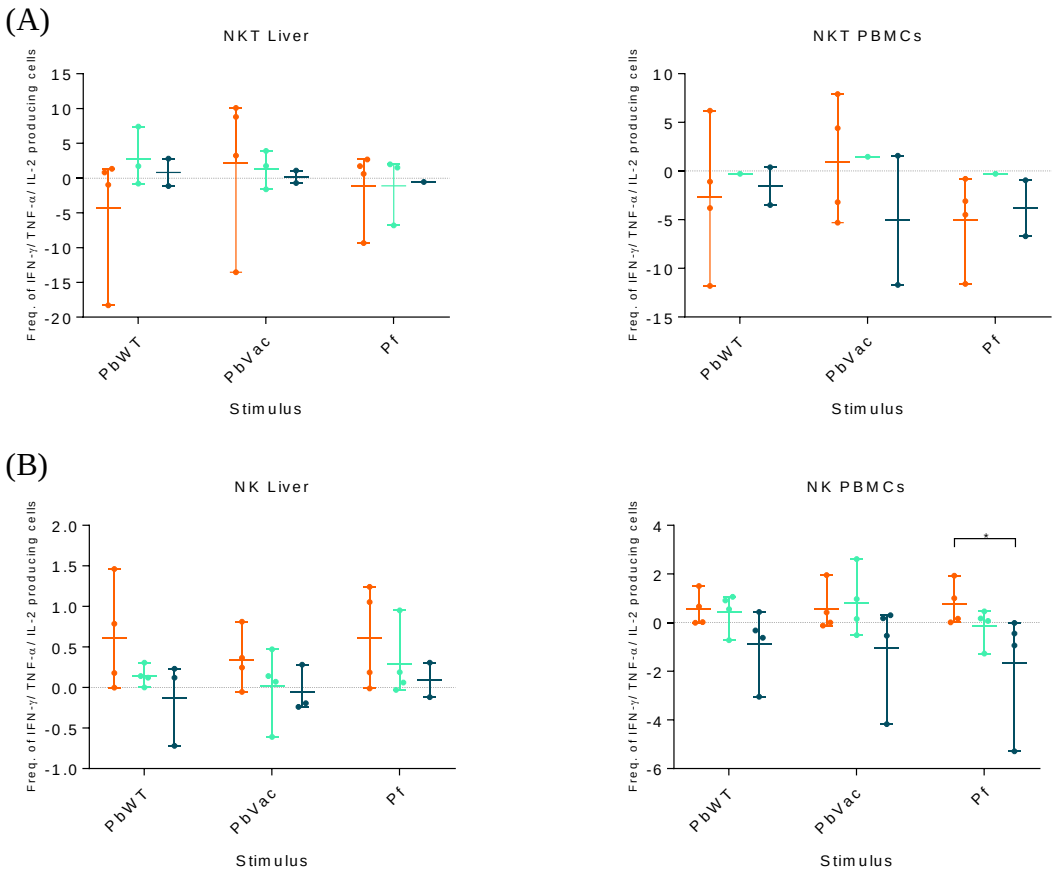


*Figure 21*- Analysis of NKT (A) NK (B) specific response in liver and PBMCs. Cytokine production *is presented as a result of the production of any of the three cytokine (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gatting strategy used it was the same as to analyze the phenotype*. Statistical significance was assessed usiang Mann-Whitney non-parametric tests

Finally, the stimulation potential of total $T_{RM}$ cells from the liver, as well as of CD4+ and CD8+ T cell populations, were analysed. Of these populations, only CD4+

$T_{RM}$ cells showed an increase in cytokine production, that was specific to *Pb*WT-immunized animals and observed against all stimulations tested.

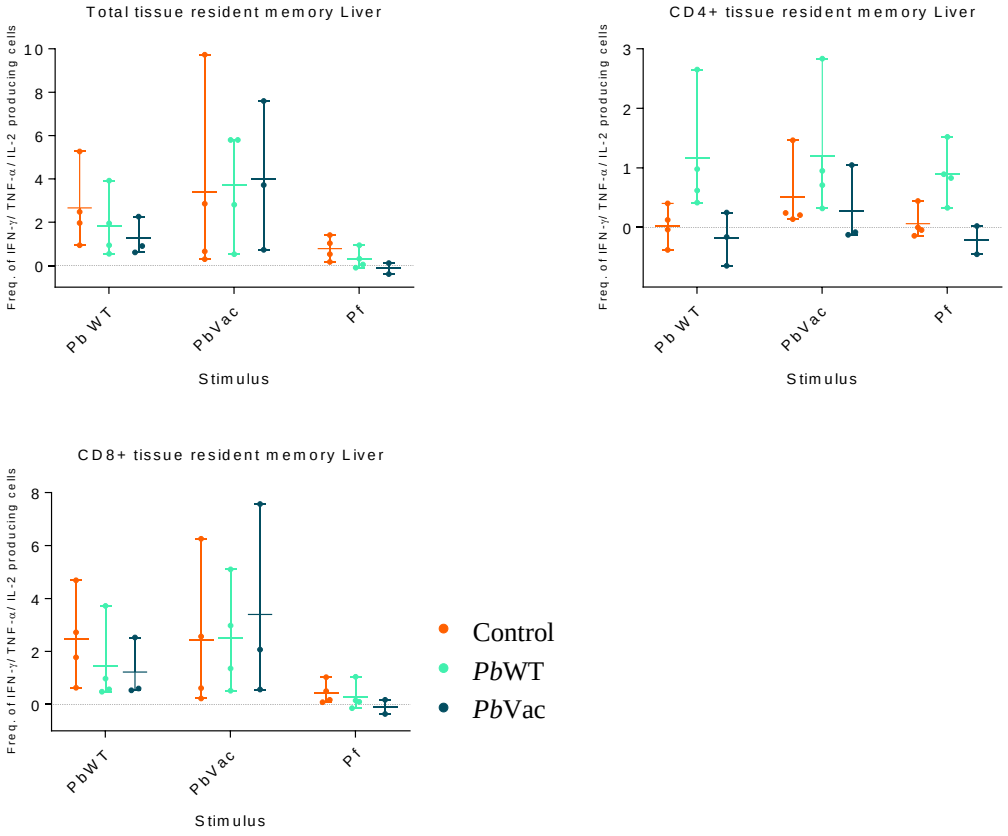


*Figure 22*- Analysis of total, CD4+ and CD8+ $T_{RM}$ specific response in liver and PBMCs.. Cytokine production *is presented as a result of the production of any of the three cytokine (IFN-γ or IL-2 or TNF-α)in a frequency of parent to which the frequency of the same unstimulated animal was subtracted.. The gatting strategy used it was the same as to analyze the phenotype.* Statistical significance was assessed usiang Mann-Whitney non-parametric tests.

# 5. Discussion

Malaria remains one of the most severe health problems worldwide, causing high morbidity and mortality in the regions where it is endemic. An effective vaccine would help close the gap left by other malaria control interventions and would be an essential tool in malaria control. Therefore, the need to develop an efficient vaccine for this disease is urgent. Nevertheless, this achievement is quite challenging due to the variability of the parasite and the lack of knowledge about the biology and immune response caused by it.

IMM's Prudêncio Lab proposed a new vaccination platform against human malaria, *Pb*Vac, in which the rodent parasite, *P. berghei*, expresses the *Pf*CS antigen. This vaccine candidate constitutes a safe "naturally attenuated" Wsp vaccine with the aim to promote humoral responses and cross species cellular immune responses that will protect against a subsequent *Pf* infection. This thesis aimed to analyse the immune responses generated by *PbVac* immunization of rhesus monkeys, which are phylogenetically close to humans.

Most vaccines work by inducing B lymphocytes to produce specific antibodies. It's the case of *Pf*CS-specific antibodies in RTS,S[112], and the primary response to CPS immunization and RAS protection, in which antibodies interfere with sporozoite motility, hepatocyte invasion and development[109]. We analysed the humoral response induced by *Pb*Vac and *Pb*WT in rhesus monkeys, as compared to non-immunized animals, and confirmed the increase in total IgG titers against *Pb*Vac and *Pb*WT sporozoites upon either immunization. In contrast, *Pf* sporozoites were only recognized by antibodies generated in *Pb*Vac-immunized but not in *PbWT*-immunized monkeys. Since *Pb*Vac and *Pb*WT differ only in the presence of *Pf*CS in the former, we may conclude that *Pb*Vac induced the generation of anti-*Pf*CS antibodies, which were able to recognize *Pf*. This is in line with results involving *Pb*Vac immunization of NZW rabbits, in which increased IgG titers anti-*Pf*CS were reported[101]. Importantly, the increase in IgG titers anti-*Pf* sporozoites supports the induction of humoral responses against *Pf* sporozoites in *Pb*Vac-immunized monkeys. It would be interesting to study the *in vitro* ability of the antibodies generated by *Pb*WT or *Pb*Vac immunization of monkeys to inhibit human hepatocyte invasion by *Pf* sporozoites, in order to understand whether humoral immunity would be functional against *Pf* infection

A great advantage of this pre-clinical study is the possibility of analysing tissues, such as the spleen and the liver, a target organ of *Plasmodium*, which is not possible in humans, as well as to compare them with immune responses measured in the blood compartment. We performed an extensive analysis of the dynamics of cellular immune populations upon immunization with either *Pb*WT or the genetically modified parasite *Pb*Vac in order to assess possible cellular changes.

We found a decrease in the frequency of blood monocytes with immunization, although this was not statistically significant. One possible explanation for this decrease is that monocytes migrated into tissues and differentiated into macrophages, a population that was not analysed in the current study.

Interestingly, we found a decrease in circulating ILCs with immunization and when comparing immunized and non-immunized animals at the last time point. This could be explained by migration of ILCs from the periphery to tissues such as the liver and spleen, where they are widely distributed. However, since we also found decreased frequency of ILCs in these organs with immunization, we may speculate that they may have migrated to other tissues, such as the skin, or even an increase in apoptosis, as has been reported for ILC1s by Ng et al.[120]

Although NKT cells do not appear to have a clear physiological role against pre-erythrocytic stages of malaria, their association with a diminished humoral response in immunization of CD1d-deficient mice and an inhibitory effect against the development of liver stages of malaria *in vivo* have been reported [121]. In our study we observed a large distribution of NKT cell frequency both in the liver and spleen cells, but no statistically significant changes in any of the compartments analyzed.

Activation and expansion of γδ T cells have been reported in the blood of *Pf*-infected individuals up to 4 weeks after vaccination[122] and *Plasmodium*-infected animals, as well as after RAS vaccination, where they are essential for induction of sterile protection[123]. In contrast, immunization of rhesus monkeys with *Pb*Vac or *Pb*WT parasites resulted in a decrease in γδ T cell frequency that was significant in the spleen. Although structural and cellular changes in malaria infection are known, such as changes in B cell populations and antigen presentation by APCs[124], there is a lack of data regarding

γδ T cell modulation in the spleen of *Pb*-infected mice in the literature for use as a comparative term.

CD4+ T cells have been shown to play an important role in CPS[111] as well as in RTS,S[112] immunizations, but not to be essential to mount immune responses in GAS immunization[107]. In our studies we observed an increase in CD4+ T cells within PBMCs following immunization, and this trend was also found in immunized animals when compared with non-immunized at the last time-point, both in PBMCs and in the liver. However, there is no increase in memory cells that would lead to long-term protection, but rather a decrease in these cells in the liver, although not statistically significant. We would expect immunization to increase the frequency of memory cells and not to decrease them, as is so widely achieved in different vaccines, so we do not know what could explain this observation.

It has been proposed that Treg cells could be induced as part of the immune response to vaccination and play a role in limiting the immunogenicity of malaria and HIV vaccines, where these cells are induced in natural infections to regulate the inflammatory response[125]. In this phenotypic analysis we found a small increase in Treg cells in the liver of immunized animals. Also Tfr cells, which have key responsibilities in the GC response[126], show an increase in frequency in the liver and spleen after immunizations. Alterations of both populations may eventually lead to a limitation of the immunogenicity of the vaccine. In its turn Tfh cells play a role in the production of long-lasting humoral immunity making it a promising cellular response to improve vaccine efficacy trough differentiation signals to GC B cell[126]. However, these immunizations do not trigger a noticeable increase in frequency in this population.

RAS immunization leads to presentation of antigens by APCs in the lymph nodes, which prime specific CD8+ T cells that then migrate to the liver[105]. IFN-γ-producing cells such as CD8+ T cells, have been identified as mediating immunity like RAS[105] and GAS[107]-based vaccines and helping to eliminate the parasite in CPS immunization[110]. CD8+ T cells are expected to be a highly active population after immunization, eliminating parasites that invade hepatocytes, and creating a repertoire of specific memory cells. Surprisingly, we found a statistically significant decrease in liver CD8+ T cells upon immunization. $T_{RM}$ cells have also been a research target after demonstration

of the capacity of rhesus monkey sporozoite immunization for generating liver $T_{RM}$ and of the result of their depletion in loss of immunity[127]. Interestingly, we found a statistically significant reduction in total $T_{RM}$ cells with immunization, which was due to the reduction in $T_{RM}$ CD8+ T cells. Although these cells are usually found at the site of infection, studies have shown they are scattered far from the site of infection, and that repeated infections increase this spread[128]. This may explain the observed decrease in CD8+ $T_{RM}$ cells, since CD8+ memory T cell cells that are most involved in the response to this parasite have spread and thus significantly decreased their frequency of controls in the target organ.

B cells are responsible for antibody production both in primary and secondary responses. Creation of a memory repertoire is an important mechanism in CPS immunization[109] and RTS,S vaccine to induce protective immunity. Although we found generation of humoral immune responses to *Pb*Vac, there were no statistically significant changes in B cell naïve and/or memory populations.

Regarding data correlation among tissues and blood, these did not occur in populations that had shown statistically significant changes, so there are highly correlated populations in blood and tissues but this did not translate into a change in population dynamics between control and immunized monkeys. In general PBMCs are not ideal for understanding what goes on in the tissues due to their poor correlation, yet they were able to give us information about NK, NKT, Treg and Tfh cells

Several vaccine trials in animal models and humans have shown the importance of the Th1-associated cytokines IL-2, IFN-γ and TNF-α in the acquisition of immunity against *Plasmodium* infection[129]. IFN-γ is a cytokine that seems to play a more relevant role, and has been described in RAS vaccines to be produced by CD4+ T cells to enhance this response or by CD8+ T cells to eliminate infected hepatocytes[105,106]. *Pf*SPZ is an example of a vaccine at an advanced stage of development where the role of those 3 cytokines was studied in a similar experiment in rhesus macaque[130] In GAS immunization, CD8+ memory cells are believed to create protection through IFN-γ-secreting $CD8^+$ T and in CPS[110] immunization IFN-γ CD8+ T cells elicit a crucial immune response. In RTS,S IFN-γ activates CD4+ T cells and induces death of infected cells, CD4-produced IL-2 activates NK cells and helps the antibody production by B cells, and induces T cell proliferation[112].

Liver and blood cells from the last time point of the experiment were stimulated with *Pb*WT, *Pb*Vac or *Pf* sporozoites in order to study specific cellular responses to each of the stimuli. Overall, we found a tendency for increased total IL-2, IFN-γ and TNF-α production in CD4+ T cells of *Pb*WT-immunized monkeys, which was statistically significant for circulating CD4+ T cells when stimulated with *Pb*Vac. In contrast NK cells had decreased cytokine in both the liver and in PBMCs, and this was statistically significant upon *Pf* stimulation in *Pb*Vac-immunized monkeys. All other populations, including TCRγδ cells and CD8+ T cells, did not show clear differences in cytokine responses to sporozoite stimulation in immunized vs. non-immunized monkeys. . In *Pf*SPZ studies was demonstrated that these attenuated sporozoites inducing a high frequency of effector memory IFN-γ–producing CD8+ T cell responses in the livers of NHPs with iv administration, but not with sc administration. The latter route of administration shows a much lower T cell response, and also antibody response, that was comparable to the responses in the human volunteers immunized sc or id.These results do not appear to correspond to any pattern observed with other vaccines. However, the variability of the results and the low number of animals analysed per group make it difficult to draw extensive and/or statistically solid conclusions on the specific cellular responses. In addition, since there was a 1 month difference between the last immunization and final organ collection, we may be analysing an inadequately late time point of cellular responses.

We can speculate that one of the reasons why *Pb*WT and *Pb*Vac immunization did not generate significant cellular immunity may be related to the lack of response by TCRγδ cells, which appear to be initial drivers of the immune response. Indeed, in mouse models of *Pb*SPZ vaccination the absence of γδ T cells impaired protective CD8+ T cell responses and ablated sterile protection. The inability of monkey γδ T cells to produce cytokines and probably to recruit other immune cells may have partially hindered other responses. The parasites are known to have been recognized by antibodies, which reached the liver and invaded the hepatocytes but for some reason these cells were unable to trigger the response and perform their functions. Have been showed such in natural infection as after vaccination that γδ T cells rapidly expand and increase in frequency, and produce cytokines, mainly IFN-γ, associated with protection. This cells also have a

cytotoxic action, present antigens, promote dendritic cell maturation, help B cells and recruit other immune cells.

Another option regards the route of administration. *Pf*SPZ experiments on rhesus monkeys have reported the ineffectiveness of sc immunization as compared to iv. Although we can argue that mosquito bite immunization is not the same as sc immunization, since there are sporozoites that are immediately able to reach blood or lymphatic vessels, it is also not the same as immunizing iv, in which it is possible to ensure that sporozoites enter the bloodstream. Mosquito bite immunization still has the caveat of not controlling the amount of sporozoites inoculated. We can thus hypothesize that the method of administration was not optimal and, therefore, did not trigger an adequate immune response. Although a humoral response has been created, we do not know whether this response could be exacerbated if the route of administration was iv.

We can also speculate that the lack of cellular responses was due to the number of mosquitoes used in each immunization. Assuming that the mosquito bites about 50 sporozoites per bite, 75 mosquitoes inoculate about 3750 sporozoites. For example, in the *Pf*SPZ vaccine, immunizations inject about 135,000 sporozoites per immunization, and in the Gf Pfb9slarp vaccine between 10 and 500,000 sporozoites were tested. From what we can see, there is still enormous potential for increasing the number of sporozoites used for immunization in order to possibly trigger a more prominent cellular response.

It would be great to be able to challenge immunized monkeys to know whether immunization translates into animal protection or attenuated parasitaemia or disease symptoms. However, this is not possible since *Pf* does not cause disease in rhesus macaques.

Or that too many days have passed since the last immunization to still be able to verify phenotypic changes in the cellular profile of the immunized monkeys, and that the low dose of immunization has not allowed memory to be created in the cells to be able to recognize the parasite antigen and release cytokines as specific response. Thus it would be possible to use isolated PBMCs at harvest during the study to also analyse their phenotype and to evaluate their specific responses to sporozoites by stimulation and to know if there was any immunization that might have led to an increase in cellular response, and if so its duration.

In the future it would still be interesting to know if an increase in immunization dose would trigger a stronger cellular immune response, so that we can know if this response is even dependent on CD4+ cells with the help of humoral immunity. Or if there was in fact no response due to the unrecognition by TCRγδ cells to sporozoites that possibly leads to the lack of immune response, and whether this pattern would continue with a second and potent experiment. Or that too many days have passed since the last immunization to still be able to verify phenotypic changes in the cellular profile of the immunized monkeys, and that the low dose of immunization has not allowed memory to be created in the cells to be able to recognize the parasite antigen and release cytokines as specific response.

# 6. Conclusion

In this work we have evaluated the humoral and cellular immune responses to *P. berghei*-based whole-sporozoite malaria vaccination in rhesus macaques.

Our results indicate that *P. berghei* parasites are able to infect rhesus hepatocytes *in vivo*, which we anticipate might equally occur in humans. Of note, *P. berghei*-based Wsp administration was safe and no breakthroughs were observed throughout the study, which are important pre-requisites for translation to the clinic.

Importantly, we found that *Pb*Vac but not *Pb*WT immunization led to the generation of circulating antibodies that recognize *Pf* sporozoites. This indicates (1) that humoral immunity against *Pf* was generated, which is an exciting result; and (2) that anti-*Pf*CSP-specific antibodies play an important role in *Pf* recognition. These results validate the use of *Pb* as a delivery platform able to efficiently generate humoral responses to the antigen(s) introduced.

In contrast to the clear humoral responses generated by *Pb*Vac, we found, in general, low cellular responses to any of the parasites employed for immunization or for *in vitro* stimulation. In the future, it would be interesting to study whether an increase in immunization dose or administration route would trigger stronger cellular immune responses.

The study presented here provides valuable information on the immune responses elicited by the *Pb*Vac vaccine candidate, and constitute an important stepping-stone for the design of a clinical trial that assesses *Pb*Vac safety and immunogenicity in humans. Most importantly, it represents another step towards the development of a much needed safe and efficient malaria vaccine.

# 7. References


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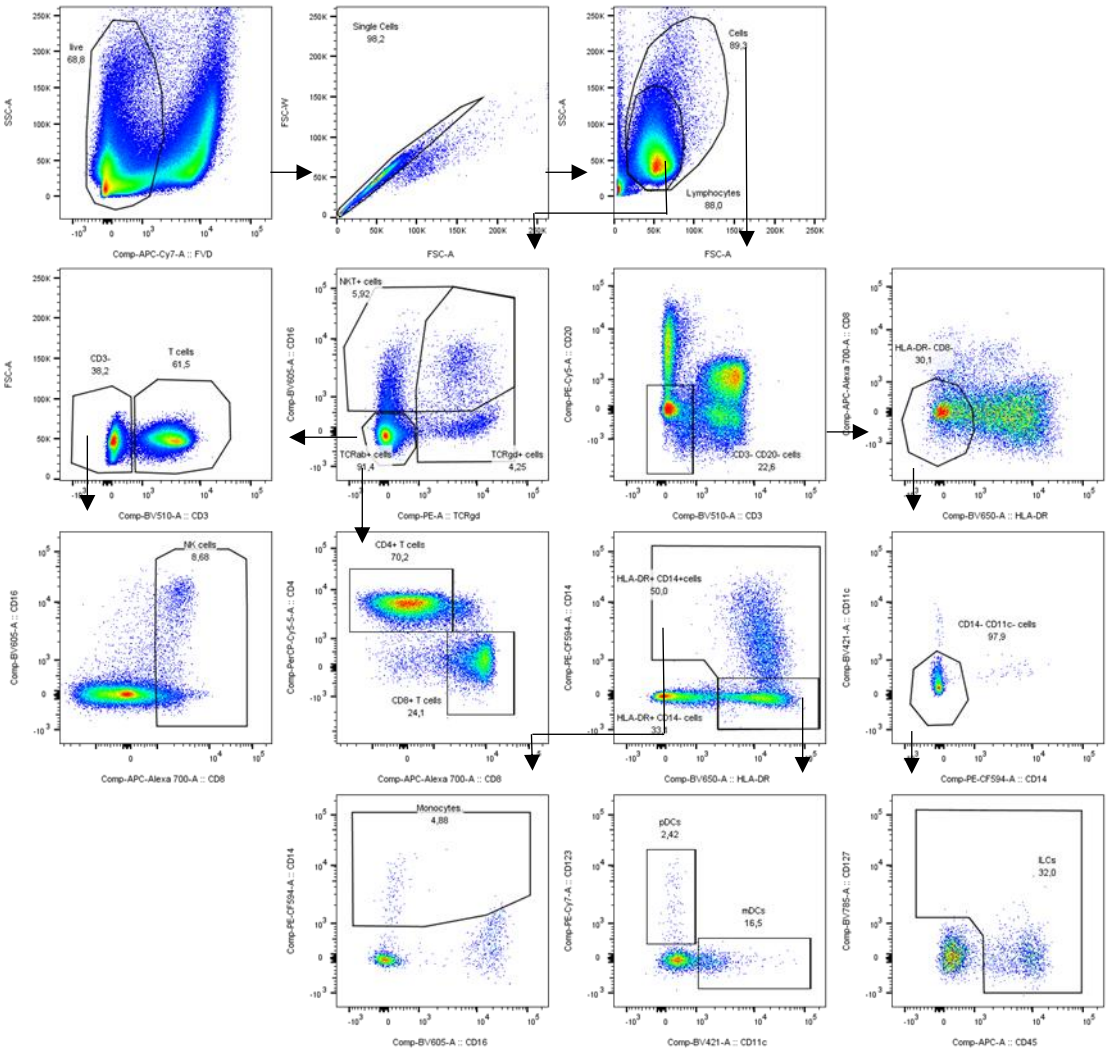
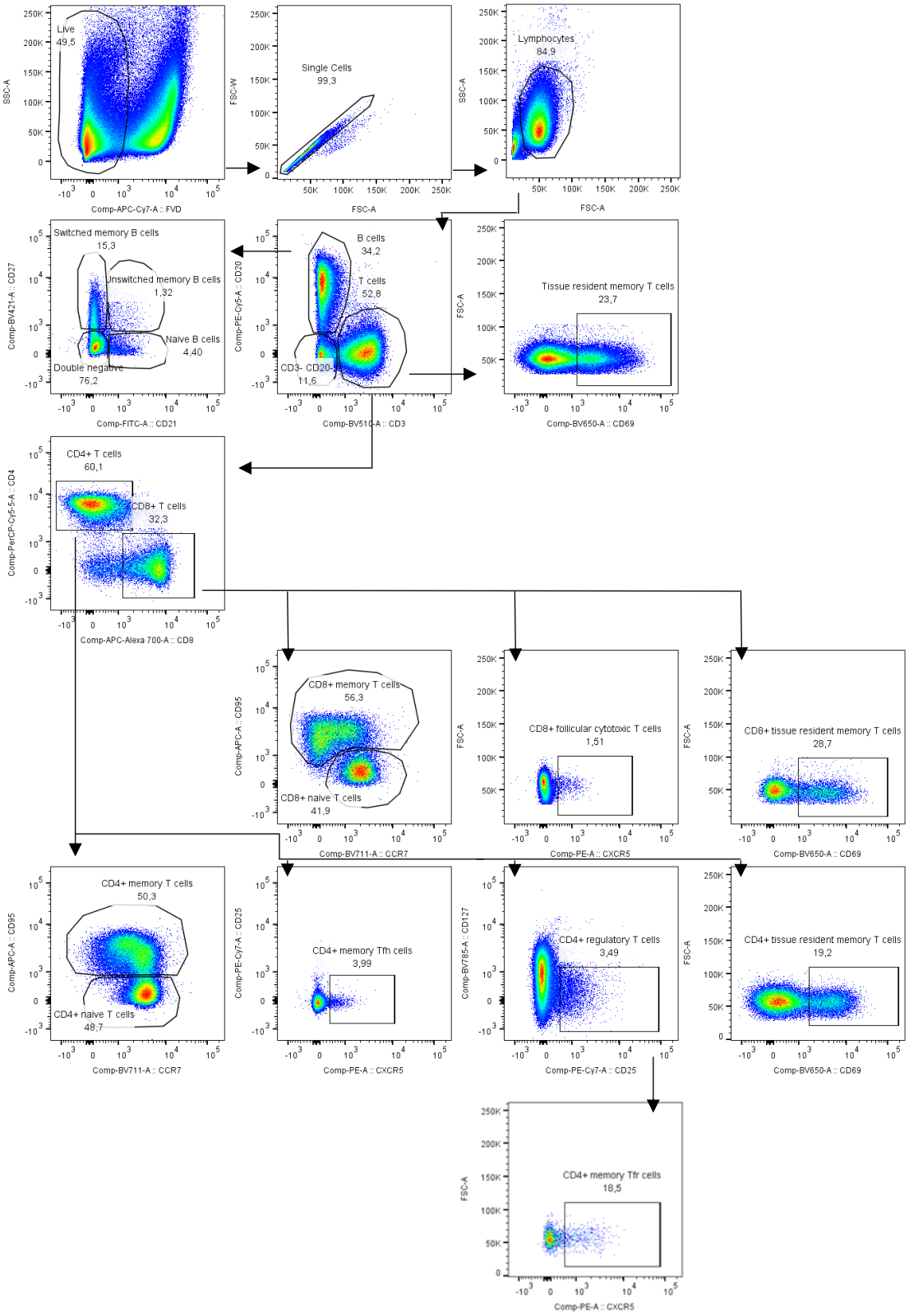
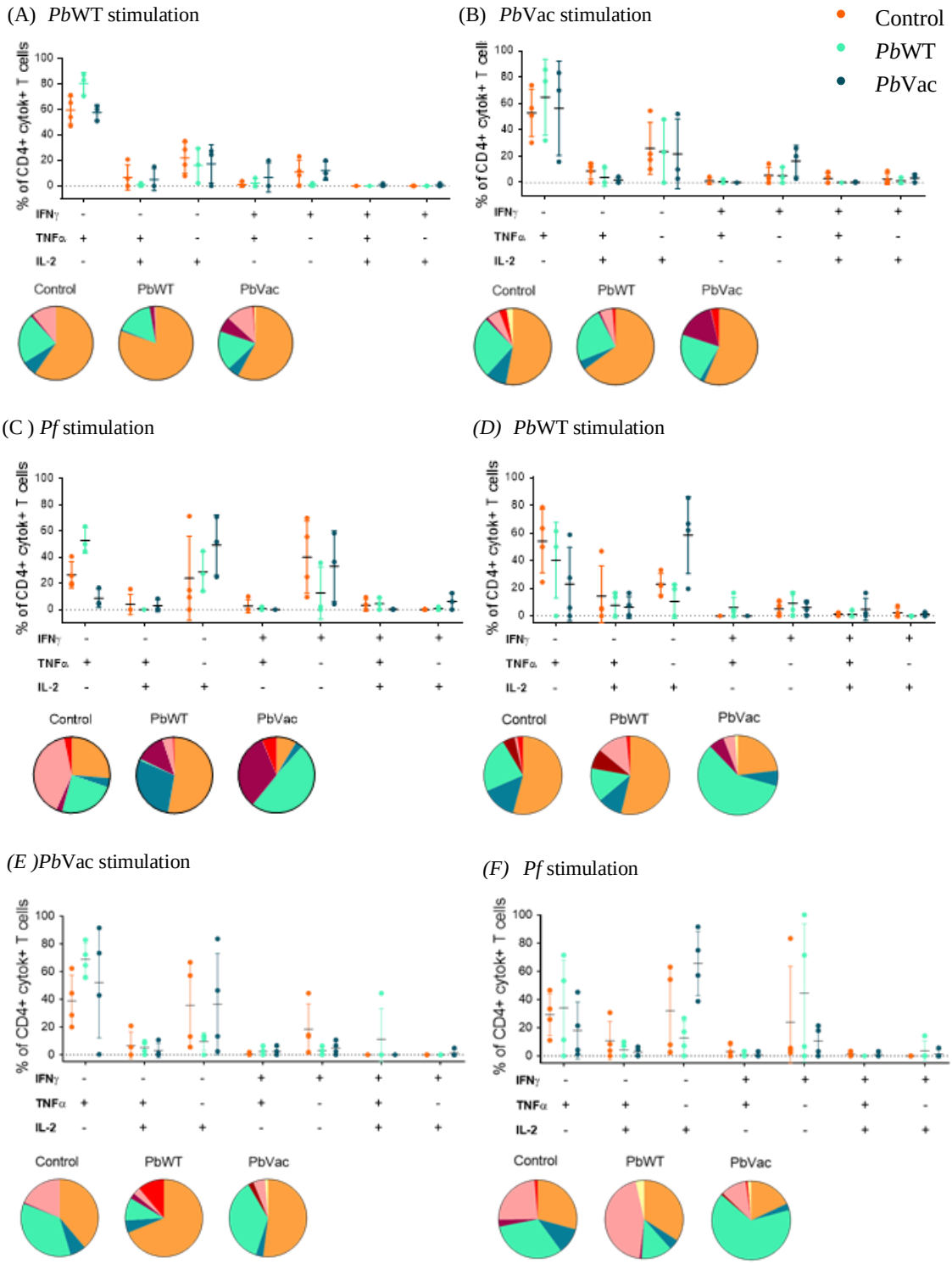
# 8. Attachments



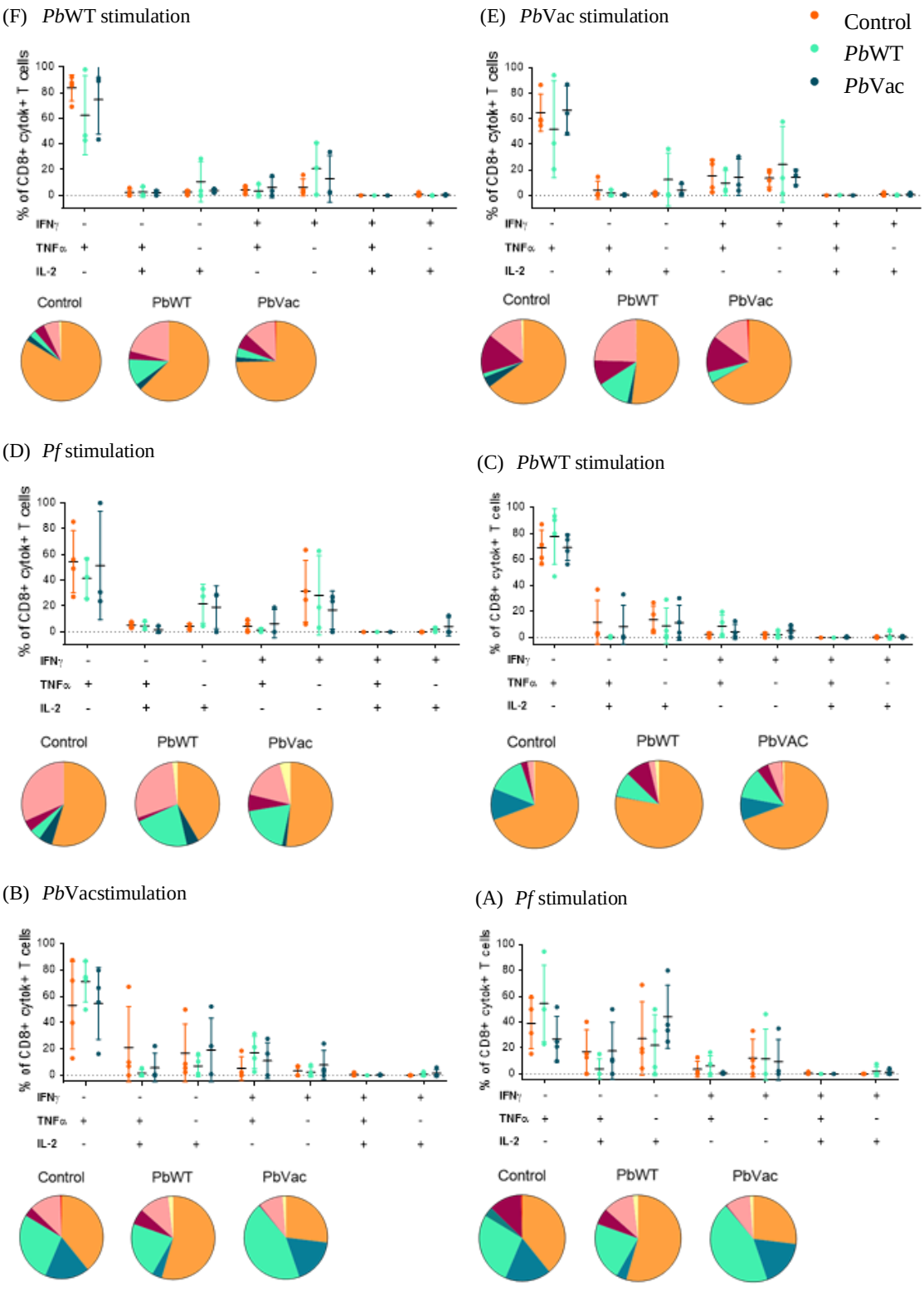

*Supplementary figure 1- Gating strategy from tube 1 of phenotypic analyses.*




*Supplementary figure 2- Gating strategy from tube 1 of phenotypic analyses.*




*Supplementary figure 3- Mono- and poly-functional cytokine production by total CD4+ T cells in the liver (A, B, C) and in PBMCs (D, E, F) upon antigen (sporozoite) encounter using an ICS assay.*




*Supplementary figure 4- Mono- and poly-functional cytokine production by total CD8+ T cells in the liver (A, B, C) and in PBMCs (D, E, F) upon antigen (sporozoite) encounter using an ICS assay.*